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Modelling human ageing: role of telomeres in stress-induced premature senescence and design of anti-ageing strategies

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Modelling human ageing: role of telomeres in stress-induced premature senescence and design of anti-ageing strategies

Rôle des télomères dans la sénescence induite prématurément par les stress et design de stratégies anti-vieillissement

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Promoteur : Dr. O. Toussaint

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Summary

Due to the duration of human ageing, researchers must rely on models such as animals and cells. Replicative senescence and stress-induced premature senescence (SIPS) are two cellular models sharing many features. Although telomeres play a major role in replicative senescence, their involvement in SIPS is unclear.

In this work, we first wanted to investigate how accurate models of ageing are. We published a new model of the evolution of human ageing, which offers a refined view of the evolution of ageing in humans and suggests that human models should be favoured. Though studying other mammals, reptiles, and birds may also be useful, we conclude that lower life forms such as yeast and invertebrates are not representative of the human ageing process.

Secondly, we wanted to elucidate the importance of telomeres in SIPS and study gene expression and regulatory networks. Using a telomerase-immortalized cell line, we found no evidence that damage specific to the telomeres is at the origin of SIPS. In our published model, neither the TGF-β1 pathway nor telomeres appear to play a crucial role in SIPS. We suggest that widespread damage to the DNA causes SIPS and propose a rearrangement of gene expression networks as a result of stress. Moreover, we advise caution in using telomerase in anti-ageing therapies since telomerase expression may alter the normal cellular functions and promote tumorogenesis.

Lastly, we published strategies to integrate the modern computational approaches to research ageing. Although we find it unlikely that a full understanding of ageing may be achieved within a near future, we argue that understanding the structure and finding key regulatory genes of the human ageing process is possible.

To Bernard Strehler, who passed away on May 13th, 2001,
hoping I will one day justify his friendship and trust.

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Abbreviations

ARCT	Ageing Research Computational Tools
ATF-2	activating transcription factor-2
ATM	ataxia telangiectasia mutated
ATP	adenosine tri-phosphate
bp	base pairs
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
cDNA	complementary DNA
CPDs	cumulative population doublings
CR	caloric restriction
Cy3	cyanine 3
Cy5	cyanine 5
DNA	desoxyribonucleic acid
g	gram(s)
G1	gap 1 (cell cycle)
G2	gap 2 (cell cycle)
GHR	growth hormone receptor
dUTP	deoxy-uridine-tri-phosphate
H_2O_2	hydrogen peroxide
HDFs	human diploid fibroblasts
hrs	hours
hTERT	catalytic subunit of human telomerase
IGF-1	insulin-like growth factor I
IMR	initial mortality rate
kbp	kilobase pairs
kcal	kilocalories
MAPK	mitogen-activated protein kinase
ml	milliliter(s)
MRDT	mortality rate doubling time
mRNA	messenger RNA
MSRA	methionine sulfoxide reductase A
mtDNA	mitochondrial DNA
O_2	oxygen
PBA	sodium 4-phenylbutyrate
PD	population doubling
pRb	retinoblastoma protein
ROS	reactive oxygen species

RS	replicative senescence
RT-PCR	reverse transcription-polymerase chain reaction
S	synthesis (cell cycle)
SA β-gal	senescence-associated β-galactosidase
S.D.	standard deviation
SIPS	stress-induced premature senescence
SM22	smooth muscle cells 22
SOD	superoxide dismutase
SOD1	Cu/ZnSOD (cytoplasmic)
SOD2	MnSOD (mitochondrial)
SV40	simian virus 40
t-BHP	tert-butylhydroperoxide
TERT	telomerase reverse transcriptase
TGF-β1	transforming growth factor-β1
TF	transcription factor
TFBS	transcription factor(s) binding site(s)
TIC	transcription initiator complex
TRF	telomeric repeat-binding factor
TSS	transcription start site
USA	United States of America
UV	ultraviolet
WRN	Werner's syndrome protein/*gene*
WS	Werner's syndrome

Introduction

CHAPTER 1: HUMAN AGEING

1.1. Definition and history

In the context of this work, ageing is defined as an intrinsic age-related process of loss of viability and increase in vulnerability (Comfort, 1964). It is possible to find cases where "ageing" has a positive connotation, such as "ageing wine". Herein, however, our focus is on the deleterious effects of ageing, what some authors call "senescence" (Finch, 1990). More precisely, we are concerned with human ageing, the changes that render human beings progressively more likely to die (Medawar, 1952).

Since ageing is a universal human feature, it is not surprising that since the dawn of civilization many have sought to avoid it. From the Babylonian epic of Gilgamesh, to Ponce de Leon seeking the "fountain of youth", countless men have dreamed of finding a way to avoid ageing. In modern times, our goals have changed little: to understand what causes human ageing and how to slow, stop, and reverse this process (de Magalhaes, 2003a). Our ultimate aim is to understand human ageing with the prospect of delaying age-related debilitation.

1.2. The ageing phenotype

The ageing process affects multiple organs and tissues (reviewed in Hayflick, 1994) and involves the progressive deterioration of virtually every bodily function (Austad, 1997a; Strehler, 1999). One outcome of ageing in many species, including humans, is the exponential increase in mortality associated with chronological age for all members of the species and first recognized by Benjamin Gompertz. The most practical way to mathematically represent the effects of ageing on mortality rates--though not entirely accurate because, for instance, mortality rates can decrease at late ages--is the Gompertz equation: $R_m = R_0 e^{\alpha t}$ where R_m is the chance of dying at age t, R_0 is the non-exponential factor in mortality, and α is the exponential parameter (Comfort, 1964; Finch, 1990; Strehler, 1999; Kowald, 2002).

Death can be unrelated to ageing. Thus, changes in parameters such as longevity, which measures how long an organism is expected to live, and lifespan, which represents maximum longevity, may or may not be related to ageing. Since ageing results in an increase in mortality, many interventions affecting longevity are often confused with interventions that affect the ageing process (Hayflick, 1994 & 2000; Kowald, 2002; Figure 1). For example, the increase in average longevity expectancy of the past century in most developed countries was due to a

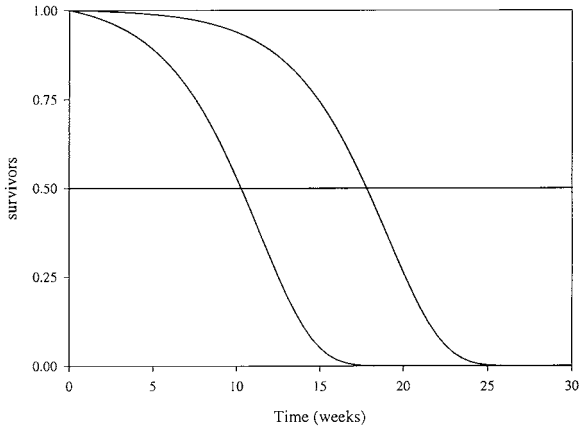

Figure 1: Mortality curves drawn based on the Gompertz equation: $R_m = R_0 e^{\alpha t}$. Survival curves for two populations with the same ageing rate ($\alpha = 0.3$), but different intrinsic vulnerabilities ($R_0 = 0.001$ and $R_0 = 0.01$). The average longevity, indicated by the 50% survivor threshold, and lifespan are clearly different. Taken from Kowald, 2002.

decrease in mortality unrelated to changes in the ageing process. Although human life expectancy is considerably higher, the rate of ageing and thus the ageing process appear unaltered since the dawn of the human species (Hayflick, 1994). In addition, many unhealthy habits may influence mortality without affecting ageing. Though there may be specific age-related pathologies, ageing is universal in humans. For instance, some evidence suggests that age-related functional decline in athletes is similar to sedentary adults (Wiswell *et al.*, 2001).

A major problem in the biology of ageing is the difficulty in measuring ageing. People can age at different paces (Finch, 1990). A frailty index can be estimated for elderly people to give a better correlation with death than age (Mitnitski *et al.*, 2002). Nonetheless, the best way to determine how aged someone is remains chronological age. Even if limitations exist in estimating ageing for an individual, through the Gompertz equation we can estimate the rate of ageing for a given population by the α parameter, which gives us the rate at which mortality increases with age (Comfort, 1964; Finch, 1990; Kowald, 2002; Figure 1). So far, all human populations have been found to age at approximately the same rate. Yet different species can age at different paces; amongst mammals, for instance, we witness about 50-fold differences in rate of ageing (Finch, 1990; Austad, 1997b).

Since ageing has multiple effects, another problem in gerontology is distinguishing causes from effects of ageing. Others have claimed before how the goal of gerontology should be to discriminate causes from effects of ageing and find the one or few physiological processes that control ageing (Medawar, 1955). Yet at present, such discrimination remains elusive.

1.3. Theories of ageing

Although it is difficult to distinguish causes from effects of ageing, many theories have emerged to explain what changes lead to ageing (for an in-depth classification, please refer to Medvedev, 1990). At present, no consensus exists over what causes ageing, what determines rate of ageing, or what changes occur in humans from age 30 to 70 to increase the chances of dying by 32-fold. Nevertheless, some theories have gathered more experimental support than others. Therefore, we based our work in those theories of ageing which have the greatest amount of experimental evidence in their favour.

1.3.1. Energy consumption hypothesis

In 1908, physiologist Max Rubner discovered a relationship between metabolic rate, body size, and longevity. In brief, long-lived animal species are on average bigger and spend fewer calories per gram of body mass than smaller, short-lived species. The energy consumption hypothesis states that animals are born with a limited amount of some substance, potential energy, or physiological capacity and the faster they use it, the faster they will die (Hayflick, 1994). Later, this hypothesis evolved into the rate of living theory: the faster the metabolic rate, the faster the biochemical activity, the faster an organism will age. In other words, ageing results from the pace at which life is lived (Pearl, 1928). This hypothesis is in accordance with the life history traits of mammals in which long lifespan is associated with delayed development and slow reproductive rates (reviewed in Austad, 1997a & 1997b).

Probably the biggest discovery so far in the biology of ageing was made in 1935, following earlier findings (Osborne *et al.*, 1917), by veterinary nutritionist Clive McCay and colleagues. They discovered they could slow ageing in laboratory rats just by making them eat less calories while maintaining normal levels of proteins, vitamins, and minerals (McCay *et al.*, 1935). This process became known as caloric restriction (CR) and appears to work in many animals, including rhesus monkeys (Bodkin *et al.*, 2003); it has been particularly well-studied in mice. From mice, we know that CR not only increases longevity and lifespan but it also postpones age-related diseases, decreases the rate of ageing, and delays development (reviewed in Weindruch and Walford, 1988; Figure 2). Doubts have for long existed on whether CR results from some technical artefact. One question is whether laboratory animals fed *ad libitum* are representative of wild animals; perhaps caloric-restricted mice represent a return to the normal food intake of wild mice (Austad, 2001a). Recent results suggest this is not the case: after correcting for body mass, *ad libitum* fed laboratory mice eat no more than wild mice. Importantly, even without using body mass corrections, food consumption in CR mice is significantly inferior to that of wild mice, so CR studies are indeed limiting the absolute and relative caloric intake when compared with wild mice (Austad and Kristan, 2003). Other doubts persist: perhaps inbred mouse stocks are not genotipically representative of wild mice and the inbreeding may favour ageing (Klebanov *et al.*, 2001 for arguments); or perhaps life-extension due to CR derives from alterations in body fat (Barzilai and Gupta, 1999; Bluher *et al.*, 2003 for arguments). Even so, CR remains the most impressive way to delay ageing in mammals, particularly since it derives from a very simple intervention.

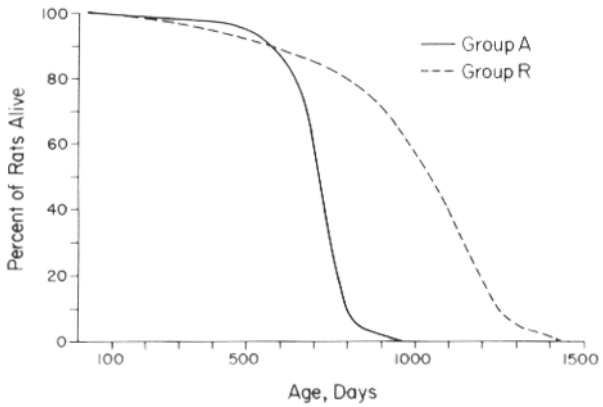

Figure 2: Survival curves for Group A and Group R rats. After six weeks of age, Group A rats (n = 115) were fed *ad libitum* while Group R rats (n = 115) were fed about 60% of the mean amount of diet consumed by Group A. Care was taken to increase the amount of vitamins in the diet of Group R so that vitamin intake was similar in both groups. Mean longevity in Group A was 701 ± 10 days and 986 ± 25 days in Group R. Maximum longevity was 963 days in Group A and 1435 days in Group R. The initial mortality rate (IMR) measures the mortality rate independently of ageing and was actually higher in Group R (IMR/year = 0.02 in group A and IMR/year = 0.029 for Group R). In contrast, the rate of ageing was different since $\alpha = 2.31$ for Group A and $\alpha = 1.31$ for Group R. Taken from Yu *et al.*, 1982. IMR and α calculations taken from Finch, 1990.

Interestingly, several genes have been identified in model organisms whose effects appear to mimic CR. The best example is probably the urokinase-type plasminogen activator. Overexpression of this gene in the brain of mice causes a decrease in appetite resulting in a 20% decrease in food consumption and body mass, and a 20% increase in longevity (Miskin and Masos, 1997). CR affects the endocrine system and, for instance, decreases the plasma levels of insulin-like growth factor I (IGF-1) and increases the levels of growth hormone (Sonntag *et al.*, 1999). At least four genes appear to result in a phenotype similar to CR in generally affecting body size, growth hormone and IGF-1, and body temperature (reviewed in Bartke *et al.*, 2001a). Yet by combining CR and mutations of one of these genes--the *Prop1* gene--, we witness an even greater increase in longevity, suggesting that distinct mechanisms may be at work (Bartke *et al.*, 2001b). Recent results suggest that although the growth hormone/IGF-1 pathway is involved in CR, other mechanisms also operate (Shimokawa *et al.*, 2003).

Although the mechanisms behind CR remain a subject of discussion, since it involves a decrease in calories, one obvious hypothesis is that CR works by delaying metabolic rates and thus serves as strong evidence in support of the energy consumption hypothesis. Body temperature is crucial to determine metabolic rate since the rate of chemical reactions rises with temperature. One common feature of animals, such as mice, rats, and monkeys, under CR is a lower body temperature (Weindruch and Walford, 1988; Ramsey *et al.*, 2000), which is consistent with the energy consumption hypothesis. On the other hand, some evidence indicates that mice under CR burn the same amount of energy as controls, suggesting they have similar metabolic rates. These studies, however, remain controversial in the way metabolic rate--in this case measured indirectly through O_2 consumption--is normalized to metabolic mass (McCarter and Palmer, 1992). One hypothesis is that CR shifts metabolic pathways (Duffy *et al.*, 1990). More recent results suggest that previous studies used unreliable estimates of metabolic mass in their calculations and indeed CR changes metabolic rates, supporting the rate of living hypothesis (Greenberg and Boozer, 2000).

Some experiments have cast doubts on the energy consumption hypothesis. For instance, mice kept at lower temperatures eat 44% more than control mice and yet do not age faster (Holloszy and Smith, 1986). Marsupials have lower body temperatures than eutherians but, on average, age faster. In contrast, birds have higher body temperatures and metabolic rates than mammals and yet, on average, age slower (Austad, 1997a).

Despite its intuitive concept, the energy consumption hypothesis does not by itself explain ageing but it can help explain some of the observations in the biology of ageing. It is true that a correlation between metabolic rates and rate of ageing can be found amongst some

mammalian species, but still much variation is found (Austad and Fischer, 1991; Austad, 1997a, 1997b; Table 1). In conclusion, the energy consumption hypothesis is one of the few pieces of the ageing process's puzzle that we know of, even though its true relevance remains unknown.

1.3.2. Free radical theory

The idea that free radicals are toxic agents was first suggested by Rebeca Gerschman and colleagues (Gerschman *et al.*, 1954). In 1956, Denham Harman developed the free radical theory of ageing (Harman, 1956; Harman, 1981). Free radicals and oxidants--such as singlet oxygen that is not a free radical--are commonly called reactive oxygen species (ROS) and are such highly reactive molecules that can damage all sorts of cellular components. ROS can originate from exogenous sources such as ultraviolet (UV) and ionising radiations or from several intracellular sources. Since oxidative damage of many types accumulate with age, the free radical theory of ageing simply argues that ageing results from the damage generated by ROS (reviewed at length in Beckman and Ames, 1998).

To protect against oxidation there are many different types of antioxidants, from vitamins C and E to enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. Briefly, antioxidant enzymes are capable of degrading ROS into inert compounds through a series of chemical reactions (Ames *et al.*, 1981). The simple existence of enzymes to prevent damage by ROS is a strong indicator that ROS are biologically important, dangerous molecules.

Most experimental evidence in favour of the free radical theory of ageing comes from invertebrates. Transgenic fruit flies, *Drosophila melanogaster*, overexpressing the cytoplasmic form of SOD, called Cu/ZnSOD or SOD1, and catalase have a 34% increase in average longevity and a delayed ageing process (Orr and Sohal, 1994). Also in *Drosophila*, expression of SOD1 in motor neurons increases longevity by 40% (Parkes *et al.*, 1998). Finally, certain long-lived strains of both *Drosophila* (Rose, 1989; Hari *et al.*, 1998) and the nematode worm *Caenorhabditis elegans* (Larsen, 1993) also have increased levels of antioxidant enzymes.

In addition to antioxidants, some enzymes catalyze the repair caused by ROS. One of such enzymes is methionine sulfoxide reductase A (MSRA), which catalyzes the repair of protein-bound methionine residues oxidized by ROS. Overexpression of MSRA in the nervous system of *Drosophila* increases longevity (Ruan *et al.*, 2002) while mice without MSRA have a decreased longevity of about 40% (Moskovitz *et al.*, 2001). Whether the ageing process is affected remains to be seen, although the results from *Drosophila* suggest that age-related decline is also delayed by MSRA overexpression. Another enzyme that repairs oxidative damage

Table 1: Variability of life history traits amongst terrestrial mammals. Notice how the lifetime energy expenditure varies almost 30-fold, which is contrary to the energy consumption hypothesis. The longevity quotient represents longevity standardized for body size. Adapted from Austad, 1997b.

Trait	***Mammalian range***
Size at birth (g)	0,005-91,000
Litter weight (as % of maternal body weight)	0.003-50
Gestation (days)	13-660
Litter size	1-16
Age at first reproduction	9 days-13 years
Maximum longevity (years)	1-120
Maximum longevity/age at first reproduction	5-122
Longevity quotient	0.14-5.39
Lifetime energy expenditure (kcal/g/lifetime)	40-1,100
Mortality rate doubling time (years)	0.3-15.4

is 8-oxo-dGTPase, which repairs 8-oxo-7,8-dihydroguanine, an abundant and mutagenic form of oxidative DNA damage. Yet contrary to the results involving MSRA, when researchers knocked out the gene responsible for 8-oxo-dGTPase, although the mutated mice had an increased cancer incidence, their ageing phenotype did not appear altered (Tsuzuki *et al.*, 2001).

One important discovery in gerontology was that targeted mutation of $p66^{shc}$ in mice increases longevity by about 30%, inducing resistance to oxidative damage, and maybe delaying ageing (Migliaccio *et al.*, 1999; Figure 3). Although the exact function of $p66^{shc}$ remains unclear, some evidence suggests it may be related to intracellular oxidants and apoptosis (Nemoto and Finkel, 2002; Trinei *et al.*, 2002; Napoli *et al.*, 2003). In another intriguing experiment, transgenic mice overexpressing the human thioredoxin gene featured an increased resistance to oxidative stress and an extended longevity of 35% (Mitsui *et al.*, 2002). Like $p66^{shc}$, thioredoxin regulates the redox content of cells and is thought to have anti-apoptotic effects (Saitoh *et al.*, 1998; Kwon *et al.*, 2003). Recently, mice with extra catalase in their mitochondria lived 18% more than controls and were less likely to develop cataracts. It remains to be seen whether these catalase-enhanced mice actually age slower (Martin, 2003). Moreover, the phenotype witnessed in a strain called senescence-accelerated mice may be related to free radical damage (Edamatsu *et al.*, 1995; Mori *et al.*, 1998).

Although ROS can have several sources, the cellular metabolism which takes place in the cell's energy source, the mitochondrion, has been receiving great attention. Some results suggest that the rate of ROS generated in the mitochondria of post-mitotic tissues helps explain the differences in lifespan amongst some animals, particularly amongst mammals (Sohal *et al.*, 1990a; Ku *et al.*, 1993; Barja and Herrero, 2000) and between birds and mammals (reviewed in Barja, 2002a). One pitfall of these studies is that technical limitations exist in measuring ROS production in isolated mitochondria. For example, none of these studies measures the levels of hydroxyl radical, the most reactive and destructive of the ROS; often, hydrogen peroxide (H_2O_2) and superoxide anion are measured since they can react to give the hydroxyl radical. Even so, such studies may not be representative of what actually occurs.

Since ROS are a result of cellular metabolism, then perhaps the free radical theory can help explain the energy consumption hypothesis. Indeed, one possible mechanism for CR is that animals under CR produce less ROS and therefore age slower (Weindruch, 1996). Of course that delayed ageing is not the only effect of CR on animals. For instance, if CR is started early in life, animals have delayed development (reviewed in Hayflick, 1994). Yet some evidence also suggests that ROS are needed for development (reviewed in Beckam and Ames, 1998), coupling ROS, development, and ageing.

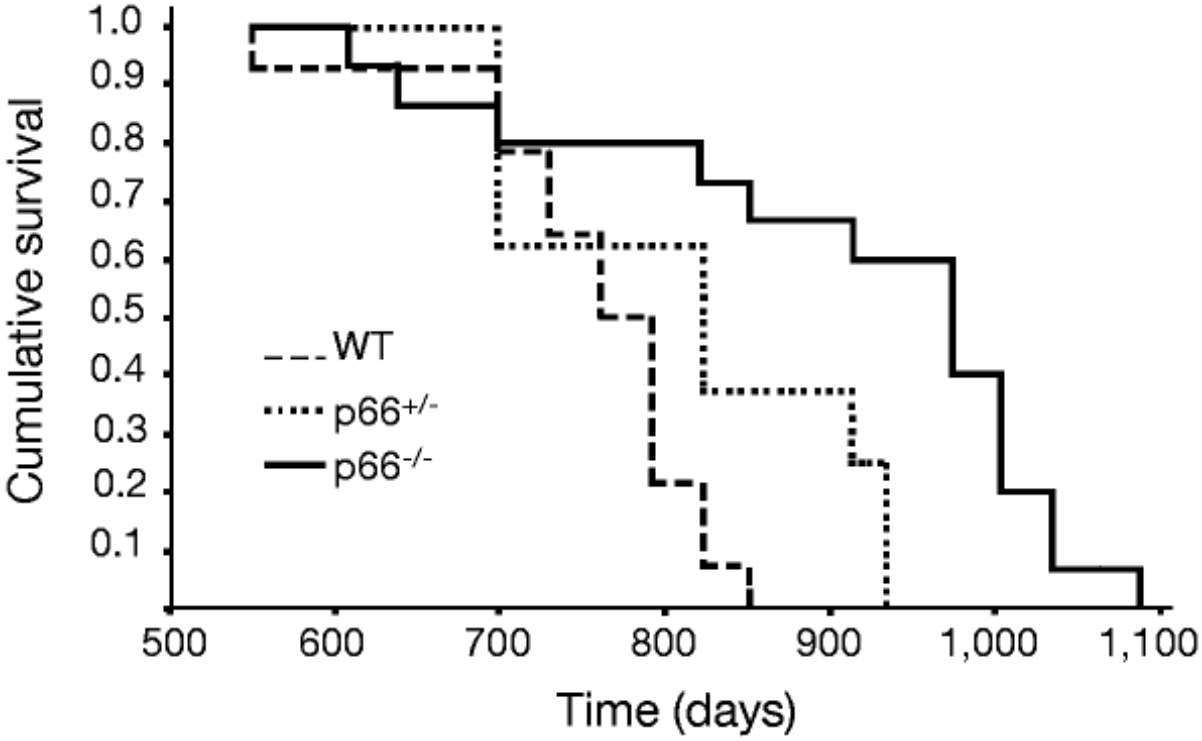

Figure 3: Cumulative survival of wild-type, $p66^{shc+/-}$ and $p66^{shc-/-}$ mice showing the prolonged lifespan of $p66^{shc-/-}$ mice. Taken from Migliaccio *et al.*, 1999.

Although there is evidence to support the role of ROS in ageing, there is also experimental evidence against it. Several attempts have been made to overexpress or knock-out antioxidants in mice, but the results have been largely disappointing. In some cases animals do not show any differences in their ageing phenotype when compared to controls (Reaume *et al.*, 1996; Ho *et al.*, 1997; Schriner *et al.*, 2000). Experiments in feeding mice antioxidants--either a single compound or a combination of compounds--were able to decrease oxidative damage and increase average longevity but none of them delayed ageing (Harman, 1968; Comfort *et al.*, 1971; Heidrick *et al.*, 1984; Saito *et al.*, 1998; Holloszy, 1998), while other studies did not conclude that feeding mice antioxidants increases longevity (Lipman *et al.*, 1998). Ubiquitous overexpression of SOD1 in mice also failed to increase longevity (Huang *et al.*, 2000). Overexpressing bovine SOD2, the mitochondrial form of SOD, also called MnSOD, in *Drosophila* slightly extends average longevity but does not delay ageing (Fleming *et al.*, 1992). In fact, recent findings suggest that the influence of SOD1 and catalase in *Drosophila* ageing may had been overestimated because they only took into account short-lived strains (Orr *et al.*, 2003). Inactivation of peroxiredoxin 1, an antioxidant enzyme, shortens longevity in mice but these do not appear to age faster but rather develop more tumours and a severe haemolytic anaemia probably derived from protein oxidation in erythrocytes (Neumann *et al.*, 2003). These results suggest that antioxidant proteins are already optimized in mammals. Indeed, correlations between rate of ageing and antioxidant levels in mammals are, if they exist, very weak (reviewed in Finch, 1990; Sohal and Weindruch, 1996). Some studies found correlations between the levels of certain antioxidants and longevity in mammals, but failed to find any consensus (Tolmasoff *et al.*, 1980; Ames *et al.*, 1981; Cutler, 1985; Sohal *et al.*, 1990b). The way antioxidants can increase longevity but do not affect rate of ageing suggests that antioxidants are healthy but do not affect the ageing process, a bit like, for instance, fish oil can extend longevity without affecting ageing (Jolly *et al.*, 2001).

One hypothesis is that the rate of mitochondrial ROS generation rather than the antioxidant level may act as a longevity determinant (Sohal and Brunk, 1992; Barja, 2002b). Yet, for instance, one recent study in *Drosophila* found that lowering ROS leakage from the mitochondria through over-expression of the mitochondrial adenine nucleotide translocase did not result in extended longevity. The same study failed to find differences in ROS production in CR flies despite these living longer (Miwa and Bran, unpublished).

Several pathologies exist in mice and humans derived from mutations affecting the mitochondrion, which often involve an increase in ROS leakage from the mitochondrion (Pitkanen and Robinson, 1996; Wallace, 1999; DiMauro and Schon, 2003). Yet these

pathologies do not result in an accelerated ageing phenotype, but frequently diseases of the central nervous system. One example is Friedreich's ataxia which appears to result from increased oxidative stress in the mitochondria and does not ressemble accelerated ageing (Rotig *et al.*, 1997; Wong *et al.*, 1999). Deficiency of the mitochondrial complex I has been reported in a variety of pathologies such as neurodegenerative disorders (reviewed in Robinson, 1998). Cytochrome c deficiency has also been associated with neurodegenerative disorders (reviewed in DiMauro and Schon, 2003) as has selective vitamin E deficiency (Burck *et al.*, 1981). Perhaps ROS are involved in some pathologies involving post-mitotic cells, such as neurons; another alternative is that mitochondrial diseases affect mainly the central nervous system due to its high energy usage (Parker, 1990 for arguments). Interestingly, both *Drosophila* and *C. elegans* are mostly composed of post-mitotic cells, which can explain why results from these invertebrates are much more supportive of the free radical theory of ageing than results from mice.

Another recent experiment raised doubts regarding the free radical theory of ageing: knockout mice heterozygous for SOD2 showed increased oxidative damage at a cellular and molecular level but did not show significant changes in longevity or rate of ageing (Williams *et al.*, 1998; Van Remmen *et al.*, 2001; Richardson, 2002).

Although it is undeniable that ROS play a role in several pathologies, including age-related pathologies, the exact influence of ROS in mammalian ageing is undetermined.

1.3.3. DNA damage theory

The DNA, due to its central role in life, is bound to be implicated in ageing. One obvious hypothesis is that damage accumulation to the DNA causes ageing (Gensler and Bernstein, 1981 for arguments).

One of the most intriguing phenotypes in the biology of ageing is the accelerated ageing witnessed in humans and animals as a result of certain mutations. Progeroid syndromes, as they are called, are rare genetic diseases of which the two most impressive forms are Werner's (WS) and Hutchinson-Gilford's syndrome (Martin and Oshima, 2000). Both these diseases originate a phenotype that is remarkably similar to an accelerated ageing process, particularly in the case of WS (Goto, 1997; Figure 4). Though differences exist in terms of pathology, what most markedly distinguishes these syndromes is age of onset with Hutchinson-Gilford's syndrome almost exclusively affecting children while WS patients normally reach adulthood.

Werner's syndrome originates in a recessive mutation in a gene, *WRN*, encoding a RecQ helicase (Yu *et al.*, 1996; Gray *et al.*, 1997). Since WRN is unique amongst its family in also

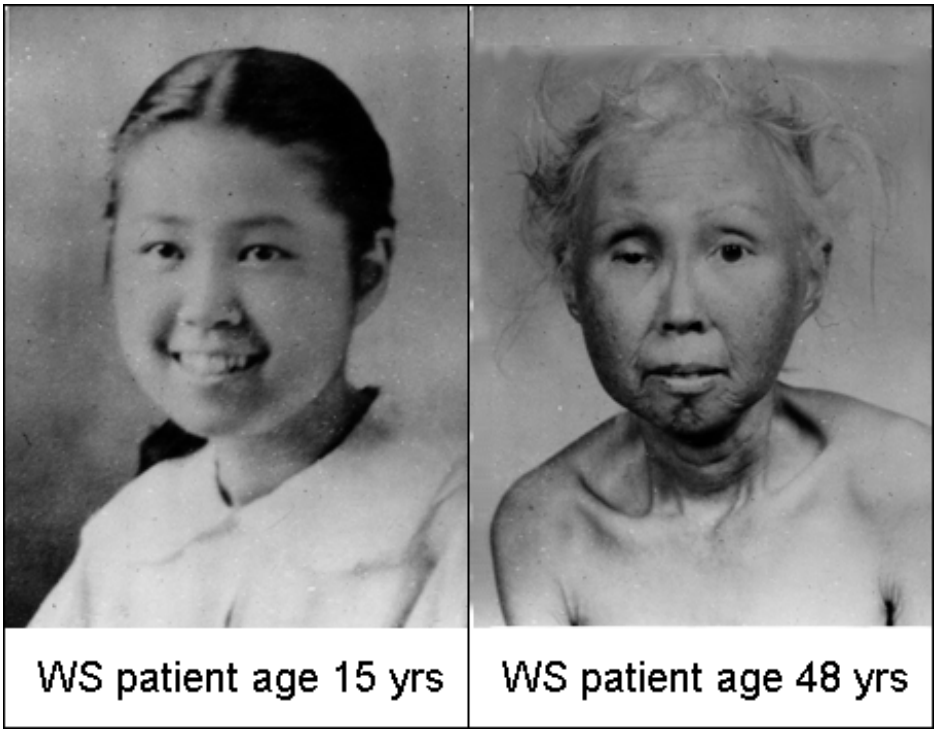

Figure 4: A patient with WS at age 15 and 48. Taken from the University of Washington Werner syndrome Home Page: http://www.pathology.washington.edu/research/werner/

possessing exonuclease activity (Huang *et al.* 1998), it may be involved in DNA repair. Although the exact functions of WRN remain a mystery, it is undeniable that WRN plays a role in DNA biology, particularly on unusual DNA structures (reviewed in Shen and Loeb, 2000; Bohr *et al.*, 2002; Fry, 2002). In fact, cells taken from patients with WS have increased genomic instability (Fukuchi *et al.*, 1989). Topoisomerases are enzymes that regulate the supercoiling in duplex DNA. WS cells are hypersensitive to topoisomerase inhibitors (Pichierri *et al.*, 2000a & 2000b). As such, WS is an indicator that alterations in the DNA over time play a role in ageing.

As with WRN, the protein whose mutation causes Hutchinson-Gilford's syndrome is also a nuclear protein: lamin A/C (Eriksson *et al.*, 2003). Recent results also suggest that some atypical cases of WS may be derived from mutations in lamin A/C (Chen *et al.*, 2003a). The exact functions of lamin A/C remain unknown, but it appears to be involved in the biology of the inner nuclear membrane. Further evidence suggests that the DNA machinery is impaired in Hutchinson-Gilford's syndrome (Wang *et al.*, 1991; Sugita *et al.*, 1995), again suggesting that changes in the DNA are important in normal ageing.

Other progeroid syndromes exist, though the classification is subjective. For example, Nijmegen breakage syndrome, which derives from a mutated DNA double-strand break repair protein (Carney *et al.*, 1998; Matsuura *et al.*, 1998; Varon *et al.*, 1998), has been considered as progeroid (Martin and Oshima, 2000). Cockayne Syndrome Type I may also be accelerated ageing and the protein involved participates in transcription and DNA metabolism (Henning *et al.*, 1995). Murine accelerated ageing syndromes have also been implicated in DNA repair such as the mouse homologues of xeroderma pigmentosum, group D (de Boer *et al.*, 2002), ataxia telangiectasia mutated or ATM (Wong *et al.*, 2003), p53 (Donehower, 2002; Tyner *et al.*, 2002; Cao *et al.*, 2003), and Ercc1 (Weeda *et al.*, 1997).

It appears certain that DNA mutations and chromosomal abnormalities increase with age in mice (Martin *et al.*, 1985; Dolle *et al.*, 1997; Vijg, 2000; Dolle and Vijg, 2002) and humans (for example: Esposito *et al.*, 1989). Yet it is impossible to tell whether these changes are effects or causes of ageing. In addition, there is no consensus as to what type of DNA changes is crucial in ageing. Correlations have been found between DNA repair mechanisms and rate of ageing in some mammalian species (Hart and Setlow, 1974; Grube and Burkle, 1992; Cortopassi and Wang, 1996), though this may be an artefact of long-lived species being on average bigger (Promislow, 1994). On the other hand, mice overexpressing p48, which is important in repairing DNA damage deriving from UV radiation, had improved DNA repair mechanisms and still did not live longer (Tang *et al.*, 2000). Mice deficient in Pms2 had elevated levels of mutations in multiple tissues and yet did not appear to age faster than controls (Narayanan *et al.*, 1997).

Embryos of mice and flies irradiated with x-rays do not age faster (reviewed in Cosgrove *et al.*, 1992; Strehler, 1999), though one report argued that Chernobyl victims do (Polyukhov *et al.*, 2000). Furthermore, the common notion that germ cells have improved DNA repair mechanisms and thus avoid ageing is itself debatable (reviewed in Walter *et al.*, 2003).

One possibility is that ROS damage DNA and some evidence exists in favour of such hypothesis (Hamilton *et al.*, 2001). Yet overexpression of catalase in the nucleus did not prevent oxidative damage to DNA (Schriner *et al.*, 2000). Damage from free radicals to nuclear DNA remains an unproven cause of ageing but since ROS originate in the mitochondria, and since mitochondria possess their own genome, many advocates of the free radical theory of ageing consider that ROS damage to mitochondrial DNA (mtDNA) is more important (Harman, 1972; Linanne *et al.*, 1989; de Grey, 1997; Barja, 2002a). For example, some evidence exists that under CR oxidative damage to mtDNA is more important than oxidative damage to nuclear DNA (reviewed in Barja, 2002a). On the other hand, the mtDNA is maternally inherited in mammals (Birky, 1995 & 2001; Taylor *et al.*, 2003) and yet no evidence exists that the heritability of longevity is different between males and females. At present, and despite contradictory evidence in favour (Khaidakov *et al.*, 2003 for arguments) and against the theory (Rasmussen *et al.*, 2003 for arguments), current technology does not appear capable of assessing the true relevance of damage to mtDNA in ageing (Lightowlers *et al.*, 1999; DiMauro *et al.*, 2002). Therefore, and although mtDNA may play a role in age-related diseases, its role in ageing remains unproven.

In conclusion, changes in DNA over time may play an important role in ageing, but the essence of those changes remains to be determined.

1.4. Evolutionary theory of ageing

Since ageing increases vulnerability and ultimately leads to the death of organisms, it is apparently in contradiction with Darwin's evolutionary theory. After all, how can evolution favour a process that, as happens in practically all mammals (reviewed in Finch, 1990), gradually increases mortality and decreases the reproductive capacity?

Although the oldest written argument on the evolution of ageing is the work of Russel Wallace, the problem of how ageing evolved was first debated by August Weismann (Weismann, 1891). Weismann's first hypothesis was that ageing evolved to the advantage of the species, not the individual, a theory known as "group selection". Yet he later dropped such ideas and since arguments in favour of group selection are scarce, modern evolutionary theory of ageing disregards group selection. Weismann then suggested that ageing evolved because

organisms that segregate germ and soma must invest additional resources to reproduce instead of maintaining the soma, and this renunciation of the soma results in ageing. Weismann's ideas were later comprised by Thomas Kirkwood to become the "disposable soma theory", which states that organisms must reach a balance between the resources they invest in soma maintenance and reproduction (Kirkwood, 1977). A consequence of the disposable soma theory is that ageing occurs due to the accumulation of damage during life and that multiple mechanisms contribute to ageing (Kirkwood and Austad, 2000).

Drawing on the theories from Weismann and others, Sir Peter Medawar developed the key idea in the evolutionary theory of ageing. His basic observation was that the force of natural selection declines with age (Medawar, 1952). Since all organisms eventually die of diseases, accidents, predation, etc., genes beneficial early in life are favoured by natural selection over genes beneficial late in life. For example, imagine a species with an average longevity of 2 years whose organisms reach puberty at age 1. There is little evolutionary advantage in having beneficial genes at age 10 because only a small fraction (4%) of the population will reach that age. On the contrary, genes that are beneficial at age 1 will be selected by evolution. Following the same reasoning, a gene that kills organisms at age 20 will have little impact on organisms bearing it since few (<0.2%) will reach such advanced ages. In other words, the greatest contribution to create a new generation comes from young, not old organisms and so the power of natural selection fades with age, making it possible for hazardous late-acting genes to exist (reviewed in Hamilton, 1966; Charlesworth, 2000).

Another important work was that of George Williams in which he strongly defended antagonistic pleiotropy. Since natural selection is weaker at later ages, then perhaps genes exist that are beneficial at earlier ages but harmful at later ages. These genes with opposite effects are called pleiotropic genes (Williams, 1957).

Therefore, evolutionary theory of ageing proposes two models for how ageing can evolve. One derives from Medawar's ideas in which genetic drift and mutation accumulation lead to the loss of late-acting beneficial genes or to the appearance of late-acting harmful genes. In Williams's model, ageing evolves due to the pleiotropic effect of genes that are beneficial early in life and then harmful at late ages. Some results from *Drosophila*, however, suggest that Medawar's theory of mutation accumulation prevails over Williams's antagonistic pleiotropy hypothesis (Charlesworth *et al.*, 1996; Hughes *et al.*, 2002), although antagonistic pleiotropy should not be completely disregarded (Rose *et al.*, 2002a).

One way to look at animal life history and ageing is through the concepts of *r* and *K* selection that were formally proposed by Robert MacArthur and Edward Wilson (MacArthur and

Wilson, 1967). In brief, *r* selection is the density-independent component of natural selection, which in practice refers to reproductive rate, while *K* selection is density dependent, referring to the biggest population resources can sustain. Organisms in hazardous environments will maximize reproduction and thus be *r*-selected while organisms in non-hazardous environments will maximize their performance under crowded conditions and thus be *K*-selected. Therefore, *r*-selection will favour rapid development, small body sizes, and a short lifespan while *K*-selection will favour delayed development, larger body sizes, and a longer lifespan (Pianka, 1970; Austad, 1997b). Amongst mammals (Table 1), humans, whales, or elephants are *K*-selected while mice and voles are *r*-selected.

Evolutionary theory of ageing is supported by significant experimental evidence. In two classical experiments, researchers were able to delay ageing in *Drosophila* by only allowing older flies to reproduce (Luckinbill and Clare, 1985; Rose, 1991). This way, the force of natural selection would no longer decrease with age. Also in accordance with the theory, Steven Austad observed that opossums living in a predator-free island aged slower and reproduced later than the same species on the more hazardous mainland (Austad, 1988; Austad, 1997a).

Although Medawar suggested that ageing was controlled by a few, key physiological processes (Medawar, 1955), modern evolutionary theory of ageing argues that ageing is multifactorial (Rose, 1991; Kirkwood and Austad, 2000). Hence, it was a surprise the way single gene knock-outs could delay ageing in animals (reviewed in Tower, 2000; Johnson, 2002). In addition, some genetic manipulations can delay ageing while not affecting reproduction (Dillin *et al.*, 2002; Marden *et al.*, 2003), which was also unexpected.

One of the most intriguing phenotypes in the biology of ageing comes from animals which appear not to age, also called animals with "negligible senescence". Studies conducted both in captivity and in the wild have shown that several species of fishes, amphibians, and reptiles, to name just vertebrates, fail to show signs of ageing (reviewed in Finch, 1990). Of course that these animals have only been studied for a limited amount of time, but even so it is surprising to find that in a 50-year study, Blanding's Turtles could increase both survivorship and reproductive output with age (Congdon *et al.*, 2001). Therefore, although evolutionary theory of ageing offers a theoretical background that explains many observations, it cannot, as it stands, be used to make predictions on the biology of ageing (Le Bourg, 2001 for arguments).

1.5. Models of human ageing

One major difficulty in studying human ageing is its duration, particularly because researchers themselves are ageing and have a limited longevity. In order to test the theories of ageing, it is crucial to have adequate models. Only since the human ageing process takes decades to develop, it is almost impossible to study it *in vivo*. Therefore, scientists have to resort to models and then extrapolate data from these different models into human ageing.

Animal models are a popular choice amongst gerontologists and much of what we know about ageing today derives from them. The short life cycles of some animals, such as *Drosophila* or *C. elegans*, makes them easy subjects of study and even mice have been extensively used in gerontology. Unicellular organisms, most notably the yeast *Saccharomyces cerevisiae*, have also been widely used to research ageing and many mutations have been found that appear to participate in yeast ageing (reviewed in Jazwinski, 2001).

The employment of inadequate models for the study of human ageing can be catastrophic for research since it can shift the focus of gerontology to pathways that, though relevant in a certain model, may be irrelevant in human ageing. Yet it can be very difficult to tell whether an organism is representative of human ageing or not. For example, the male of Australian mice, *Anthecinus stuartii*, has a bizarre phenotype called "Big Bang reproduction". Once a year, during mating season, the males of this species have such an increased libido that they are unable to eat and eventually die of sexual stress (reviewed in Gosden, 1996). Breeding only once is called semelparity but, although it leads to death, it is much different from the gradual waning of humans and so *Anthecinus* do not appear good models of human ageing. Yet there is no *a priori* reason to expect other gradually ageing organisms to age for the same causes as humans.

Some mechanisms of ageing are seen across different species. For instance, CR appears to work in a variety of organisms. On the other hand, human physiology can be much different than that of model organisms. *Drosophila* and *C. elegans* are mostly composed of post-mitotic cells, which means that not only they do not have cancer but raises doubts on how valid results obtained in these animals when extrapolated to humans are. In addition, the phenotypes of ageing in yeast and humans are totally unalike (Gershon and Gershon, 2000a). It has been argued that similar mechanisms operate across many species (Longo, 1999; Longo and Fabrizio, 2002) while other gerontologists argue that there are mechanisms of ageing common to all species and mechanisms that are unique (Martin *et al.*, 1996). At present, it is impossible to tell for sure.

Since CR affects the growth hormone/IGF-1 axis, one hypothesis is that CR decreases the stimulus for cellular replication (Sonntag *et al.*, 1999). Therefore, another widely used model

involves cells, such as human cells. These have the advantage of allowing researchers to study human processes but the disadvantage that the difference between cells *in vivo* and *in vitro* may bias the results. For example, we have learned much from studying cells from patients with WS--itself a model of normal human ageing--as well as from studying the purified WRN (reviewed in Bohr *et al.*, 2002; Fry, 2002). In addition, cells from mice lacking MSRA are more susceptible to ROS (Moskovitz *et al.*, 2001), demonstrating how it is possible to reconcile and integrate results from models of ageing to fit our theories. Nevertheless, *in vitro* results are often not totally representative of what happens *in vivo* (for example: Mondello *et al.*, 1999), as will be developed in chapter 2.

The validity of each model of human ageing has always been a subject of much debate and will be thoroughly considered in this work.

CHAPTER 2: REPLICATIVE SENESCENCE AND STRESS

2.1. Hayflick's limit

In 1961, and in contradiction to what was thought at the time, Leonard Hayflick and Paul Moorhead discovered that human cells derived from embryonic tissues can only divide a finite number of times in culture (Hayflick and Moorhead, 1961). They defined the stages of cell culture as three phases: Phase I is the primary culture, when cells from the explant multiply to cover the surface of the culture flask. Phase II represents the time when cells divide in culture, which will be described briefly. Once cells cover a flask's surface, they stop multiplying. For cell growth to continue, the cells must be subcultivated. To do so, one removes the culture's medium and adds a digestive enzyme called trypsin that dissolves the substances keeping cells together; afterwards, growth medium is added. This growth medium contains the cells in suspension and can be divided by, for instance, two new flasks. Later, cells attach to the flask's floor and start dividing once again until a new subcultivation is required. Cells divide vigorously and can often be subcultivated in a matter of a few days. Lastly, Phase III begins when cells start dividing slower. Eventually they stop dividing at all and die (reviewed in Hayflick, 1985; Hayflick, 1994). Hayflick and Moorhead noticed that cultures stopped dividing after an average of fifty cumulative population doublings (CPDs). This phenomenon is known as Hayflick's limit, Phase III phenomenon (Figure 5), or, as it will be called herein, replicative senescence (RS).

Hayflick and Moorhead worked with fibroblasts, a cell type found in connective tissue, but RS has been found in other cell types: keratinocytes (Rheinwald and Green, 1975), endothelial cells (Mueller *et al.*, 1980), lymphocytes (Tice *et al.*, 1979), adrenocortical cells (Hornsby and Gill, 1978), vascular smooth muscle cells (Bierman, 1978; Volicer *et al.*, 1983), chondrocytes (Evans and Georgescu, 1983), etc. In addition, RS occurs in cells derived from embryonic tissues or from adults of all ages and in cells taken from many animals: mice, chickens, Galapagos tortoise, etc. (reviewed in Hayflick, 1994). Early results suggested a relation between the number of CPDs cells undergo in culture and the longevity of the species from which the cells were derived. For example, cells from the Galapagos tortoise, which can live over a century, divide about 110 times (Goldstein, 1974), while mouse cells divide roughly 15 times (Stanley *et al.*, 1976; Rohme, 1981). In addition, cells taken from patients with progeroid syndromes such as WS endure far less CPDs than normal cells (Salk *et al.*, 1981). Exceptions exist and certain cell lines never reach RS. These are said to be "immortal" and include

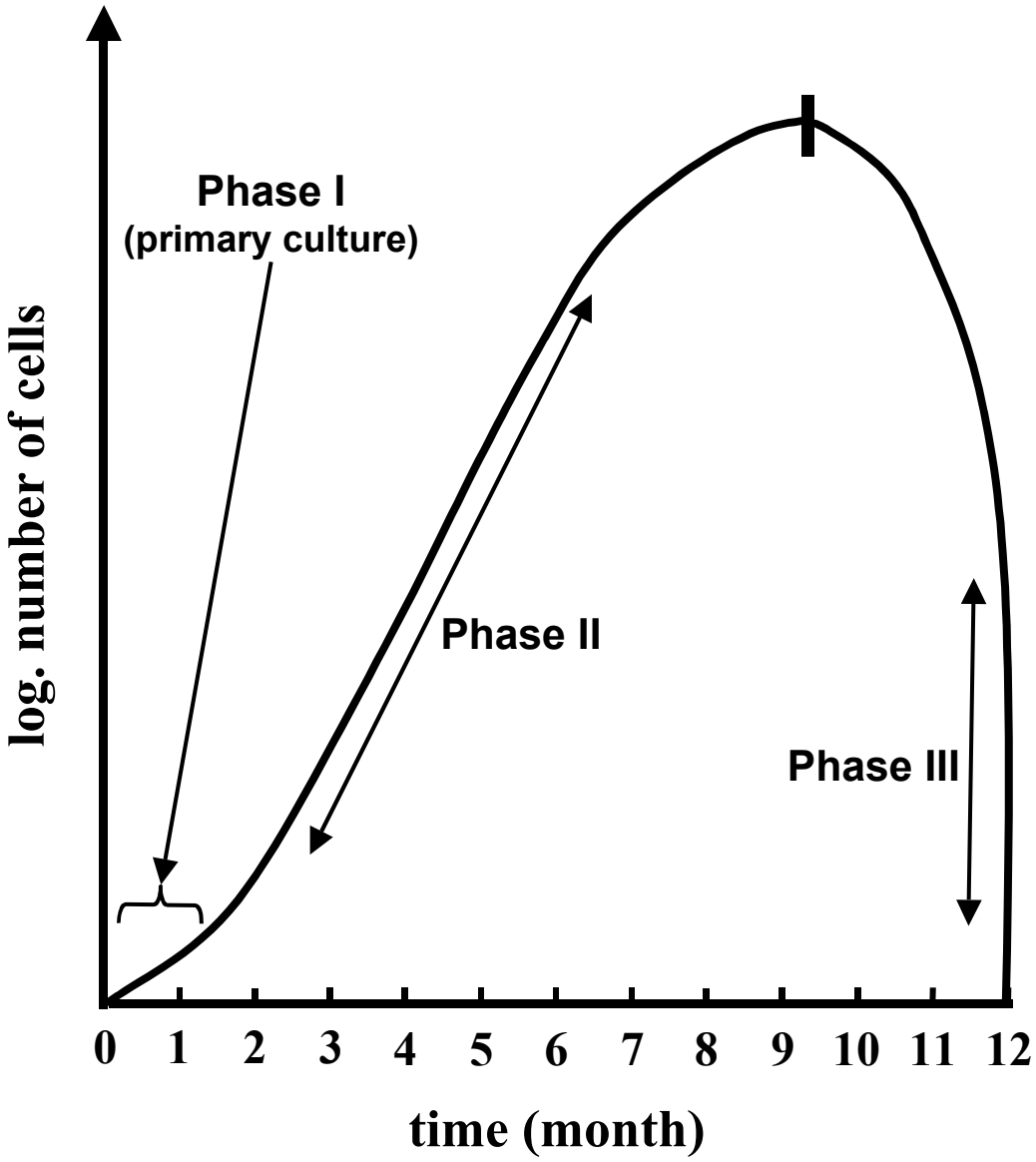

Figure 5: Representation of the three stages of cell culture. Adapted from Hayflick and Moorhead, 1961.

embryonic germ cells and most cell lines derived from tumours, such as HeLa cells (Brunmark *et al.*, 1986; Chen and Yu, 1994; Pera *et al.*, 2000). Some types of rat cells have also been claimed as capable of evading RS (Mathon *et al.*, 2001; Tang *et al.*, 2001).

Cultivating cells *in vitro* is easy and inexpensive when compared to other models of human ageing and so RS has been a widely studied phenotype.

2.1.1. Biomarkers of RS

The phenotype of RS in human diploid fibroblasts (HDFs), which were the subject of our work, is characterized by a series of features, termed "biomarkers" (reviewed in Campisi, 1999). The most obvious biomarker is growth arrest, i.e. cells stop dividing, which can be detected by [^{3}H]-thymidine incorporation. Even vigorously dividing cultures are heterogeneous and contain a percentage of senescent cells that progressively increases until cells stop dividing (Cristofalo and Sharf, 1973; Smith and Whitney, 1980); this percentage is higher in WS cells (Kill *et al.*, 1994). Senescent cells are growth arrested in the transition from phase G1 to phase S of the cell cycle (Sherwood *et al.*, 1988; Figure 6). The growth arrest in RS is irreversible in the sense that growth factors cannot stimulate the cells to divide (reviewed in Cristofalo and Pignolo, 1993), even though senescent cells can remain metabolically active for long periods of time (reviewed in Goldstein, 1990).

Another important biomarker is cellular morphology. The progressive morphological changes cells endure while they age *in vitro* were particularly well-studied by Klaus Bayreuther and colleagues (Bayreuther *et al.*, 1988a & 1988b). In brief, senescent cells are bigger and a senescent population has more diverse morphotypes than cells at earlier CPDs. In fact, a confluent senescent culture has a smaller cellular density than a confluent young culture, though this also occurs because senescent cells are more sensitive to cell-cell contact inhibition.

In 1995, Judith Campisi and her team discovered that the enzyme β-galactosidase has an abnormal behaviour associated with senescent cells, which is termed senescence-associated β-galactosidase (SA β-gal) activity. β-galactosidase, a lysosomal hydrolase, is normally active at pH 4, but in senescent cells it often happens for β-galactosidase to be active at pH 6. Both *in vitro* and *in vivo*, the percentage of cells positive for SA β-gal increases with, respectively, CPDs and age (Dimri *et al.*, 1995; Figure 7, Table 2). On the contrary, in immortal cell lines, such as HeLa cells, the percentage of cells positive for SA β-gal does not correlate with CPDs. In addition, it is possible to find a correlation between the increase in SA β-gal and the appearance of the senescent morphotypes (Toussaint *et al.*, 2000). Early reports showed that lysosomes

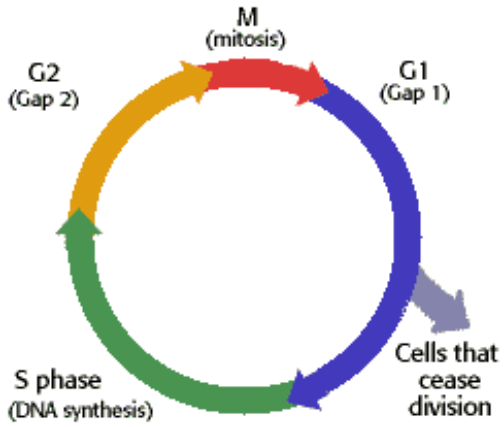

Figure 6: Stages of the cell cycle. Adapted from The Biology Project, developed at the University of Arizona: http://www.biology.arizona.edu.

increase in number and size in senescent cells (Robbins *et al.*, 1970; Brunk *et al.*, 1973). SA β-gal appears to be a result of increased lysosomal activity at a suboptimal pH, which becomes detectable in senescent cells due to an increase in lysosomal content (Kurz *et al.*, 2000). Recent results also suggest that during *in vitro* ageing increased autophagy may be associated with an increase of lysosomal mass and SA β-gal (Gerland *et al.*, 2003).

Normal human cells are diploid. Yet with each subcultivation, the percentage of polyploid cells increases (Matsumura, 1980). Mutations to mtDNA also increase with age. For example, the first identified mutation was a deletion of 4,977 base pairs (bp) in the 16,569 bp mtDNA. This deletion is observed both *in vivo* (Corral-Debrinski *et al.*, 1992; Yang *et al.*, 1994; Liu *et al.*, 1998) and *in vitro* (Dumont *et al.*, 2000a).

Senescent cells also have a decreased ability to express heat shock proteins both *in vivo* (Blake *et al.*, 1991; Fargnoli *et al.*, 1990) and *in vitro* (Choi *et al.*, 1990; Bonelli *et al.*, 1999). In addition, *in vitro* ageing makes HDFs lose *c-fos* inducibility by serum (Seshadri and Campisi, 1990).

The expression levels of several genes change during *in vitro* cellular ageing (reviewed in Cristofalo *et al.*, 1998a). Some of the most commonly used biomarkers are: osteonectin, fibronectin, apolipoprotein J, smooth muscle cells 22 (SM22), and type II α(1)-procollagen, whose expression increases in senescent WI-38 and IMR-90 HDFs (Kumazaki *et al.*, 1991; Gonos *et al.*, 1998; Dumont *et al.*, 2000a). Lastly, senescent cells also display an increased activity of metalloproteinases, which degrade the extracellular matrix (reviewed in Campisi, 1999).

Telomeres are non-coding regions at the tips of chromosomes (Figure 8). In vertebrates, they are composed of repeated sequences of TTAGGG (Moyzis *et al.*, 1988; Meyne *et al.*, 1989). During *in vitro* ageing, the telomeres shorten gradually in each subcultivation (Harley *et al.*, 1990). The same process might occur *in vivo* too (Hastie *et al.*, 1990; Lindsey *et al.*, 1991; Allsopp *et al.*, 1992). Since the telomeres are one of the main players in RS, we will focus on them in more detail in chapter 3.

2.2. The theory of stress

The pioneer in the study of stress was Hans Selye, who defined biological stress as “the non-specific response of the body to any demands made upon it” (Selye, 1973). Biological systems are constantly interacting with the environment, always under some type of stress: variations in temperature, UV radiation, emotional stress, etc. Some kinds of stress, such as mild

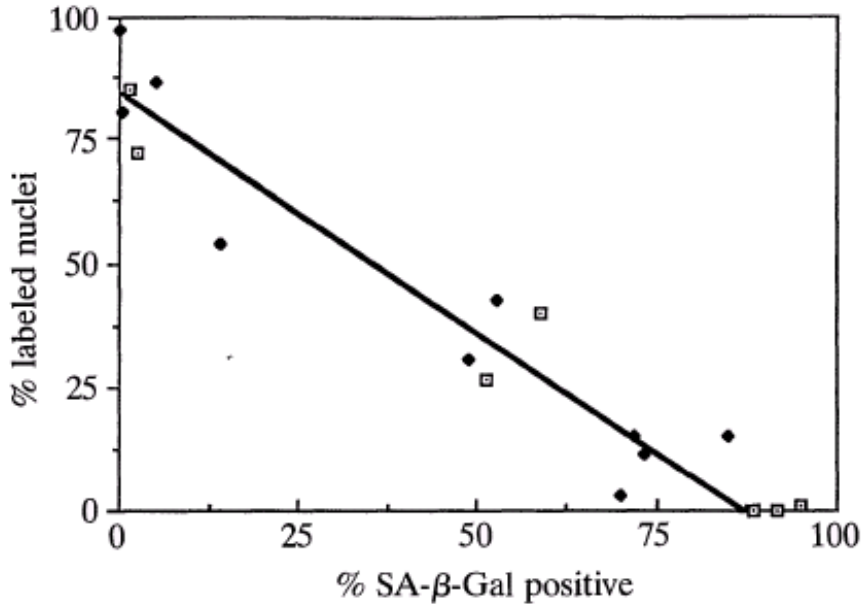

Figure 7: SA β-gal and replicative capacity during serial passage. Cells were radiolabeled with [^{3}H]-thymidine for 72 hrs, stained, and 100-500 cells were scored for labelled nuclei and SA β-gal staining. Open symbols, WI-38 cells; closed symbols, HCA2 cells. Adapted, as was Table 2, from Dimri *et al.*, 1995.

Table 2. SA-β-Gal activity in human skin from donors of different age

				SA-β-Gal staining	
Donor	Age, yr	Sex	Site	Epidermis	Dermis
Young					
1	37	♀	Cheek	+	−
2	38	♀	Shoulder	−	−
3	37	♀	Nose	±	−
4	20	♀	Lip	−	−
5	39	♀	Lip	++	−
6	31	♂	Scalp	−	−
7	31	♀	Scalp	−	−
8	38	♂	Cheek	±	−
9	33	♂	Nose	+	−
10	31	♂	Temple	±	−
Old					
1	78	♀	Nose	+++	++
2	69	♂	Temple	±	++
3	73	♀	Nose	++	++
4	76	♂	Arm	+++	+++
5	81	♀	Lip	+++	−
6	70	♀	Lip	+++	++
7	81	♂	Neck	+++	+++
8	73	♂	Temple	+++	+++
9	75	♂	Scalp	++	+++
10	90	♂	Scalp	±	+++

physical exercise, contribute to our wellness while others, such as intense emotional stress, contribute to sickness. Selye defined these two kinds of stress as, respectively, eustress, which is a "good" stress, and distress, a "bad" stress. According to the theory of stress, the initial reaction to a stressor is shock, followed by a countershock, which increases the resistance to the stressor. Yet although a stressor can contribute to wellness, the intensity or accumulation of stressors can ultimately cause sickness (Selye, 1976; Berczi, 1998).

One frequent feature of extended longevity is increased stress resistance (reviewed in Longo, 1999). Concisely, manipulations in *C. elegans* that extend longevity show a strong correlation with resistance to stress (Murakami *et al.*, 2000). In *Drosophila* too some mutations can increase longevity and augment stress resistance (Lin *et al.*, 1998a). The mutation of $p66^{shc}$ not only increases longevity in mice but also renders mouse embryonic fibroblasts more resistant to different types of stressors (Migliaccio *et al.*, 1999). As previously mentioned, mutations in MSRA can influence longevity in mice and they also influence stress resistance (Moskovitz *et al.*, 2001). In addition, avian cells are more resistant to oxidative stress than mice cells (Ogburn *et al.*, 1998), showing a correlation between longevity and cellular stress resistance. Stress resistance *in vitro* also correlates with mammalian longevity (Kapahi *et al.*, 1999). Finally, cells taken from patients with progeroid syndromes are more susceptible to stress (Gebhart *et al.*, 1988), as are senescent fibroblasts (Yuan *et al.*, 1997).

Such findings are in accordance with the disposable soma theory, since an enhanced stress resistance suggests an additional investment in soma maintenance (Kirkwood and Austad, 2000). Of course, it is not known whether stress-resistance is related to rate of ageing. Nevertheless, ageing and stress-resistance appear to be inversely related. Importantly, an association between cellular stress resistance and organismal ageing appears to exist.

2.3. Stress-induced premature senescence

Stress can have beneficial effects. Low doses of potentially toxic stressors can have a stimulatory effect: a phenomenon known as hormesis. For example, as mentioned in chapter 1.3.3., mice and flies exposed to low doses of x-rays at embryonic stages actually live longer (reviewed in Strehler, 1999). Repeated mild heat shocks appear to delay ageing in HDFs (Rattan, 1998). Yet stress can also have harmful roles. For example, *Drosophila* fed H_2O_2 at concentrations ranging from 0 to 100 mM had a shorter longevity except flies fed 10 mM which had an augmented longevity when compared to controls (Sohal, 1988).

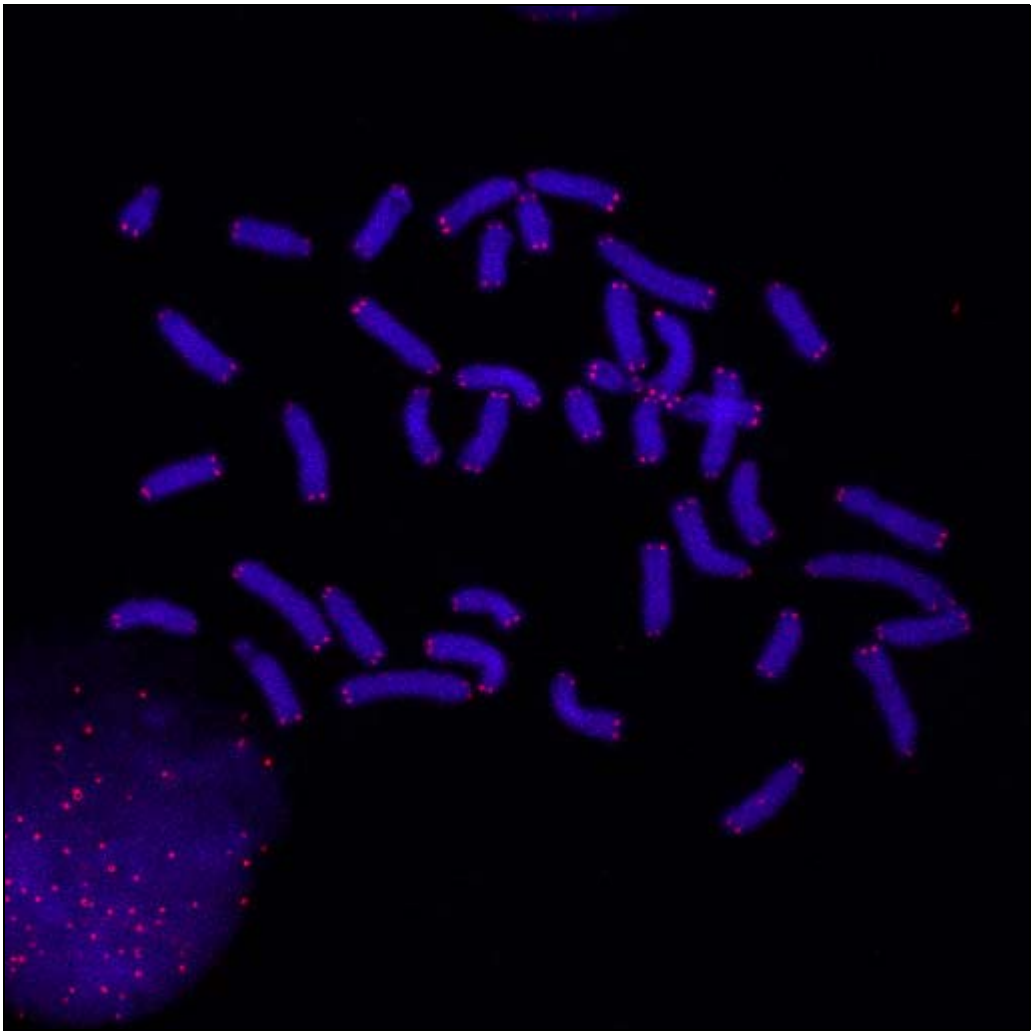

Figure 8: Human metaphase chromosomes hybridised in situ with the telomere repeat sequence. Taken from the Shay/Wright laboratory, The University of Texas Southwestern Medical Center at Dallas:
http://www.swmed.edu/home_pages/cellbio/shay-wright/intro/gallery/sw_gallery.html

One easy and inexpensive way to study the harmful effects of stress is *in vitro*. Normally, cell culture conditions include 20% oxygen (O_2) and these were the conditions initially used by Hayflick and Moorhead and most subsequent studies. When HDFs are cultured at 3% O_2, which is closer to physiological conditions, they achieve a further 20 CPDs (Chen *et al.*, 1995a). In contrast, different types of human cells cultured above 20% O_2 display a reduced growth rate and endure fewer CPDs (Horikoshi *et al.*, 1986; Michiels *et al.*, 1990; Horikoshi *et al.*, 1991; von Zglinicki *et al.*, 1995). Interestingly, the same effect is not witnessed in tumour cell lines (Saito *et al.*, 1995). In normal human cells, O_2 has been shown to accelerate growth arrest (Alaluf *et al.*, 2000). If O_2 is above 50%, it becomes cytotoxic (Horikoshi *et al.*, 1991). The way subcytotoxic stress can accelerate the appearance of the senescent phenotype in cells is another form of cellular senescence. In 1999, at the EMBO workshop of Molecular and Cellular Gerontology, Olivone, Switzerland, the term stress-induced premature senescence (SIPS) was coined (Brack *et al.*, 2000).

Depending on the dose of stressor used, a cell population will react in different ways. For instance, a high, cytotoxic dosage of a stressor causes such an amount of damage that cellular biochemical activities decrease leading to cellular death by necrosis. The level of damage sustained by cells determines whether programmed cell death, apoptosis, can unfold. If, for example, intracellular ATP depletion occurs past a certain threshold then necrosis occurs (reviewed in Lemasters *et al.*, 1999; Nicotera *et al.*, 1999). Since a cellular population is not homogeneous, the dose of the stressor will shift the percentage of cells executing each of the possible programs (Toussaint *et al.*, 2002a). In order for SIPS to occur, a precise subcytotoxic dose must be determined for each cell population (Figure 9).

In addition to O_2, other stressors--e.g. ethanol, ionising radiations, and mitomycin C--in many types of proliferative cells such as lung and skin fibroblasts, endothelial cells, melanocytes, and retinal pigment epithelial cells can induce SIPS (reviewed in Toussaint *et al.*, 2002b; Dierick *et al.*, 2003). The list of stressors that can cause SIPS is constantly growing (Table 3). Studying SIPS can have medical applications (Toussaint *et al.*, 2000 for arguments). For instance, skin HDFs repeatedly exposed to UVB undergo SIPS (Chainiaux *et al.*, 2002), which can be used to develop solar protectors and study the ageing of the skin.

Of relevance to our work are ways of inducing SIPS that instead of chronic are based on a single or repeated short exposure(s) to stressors. Namely, our focus was on damaging agents. Exposure of HDFs to a single H_2O_2 2-hour stress induces SIPS (Chen and Ames, 1994; Chen *et al.*, 1998). Another source of oxidative damage, tert-butylhydroperoxide (t-BHP), can also induce SIPS in WI-38 HDFs by repeated exposure (Toussaint *et al.*, 1992; Dumont *et al.*,

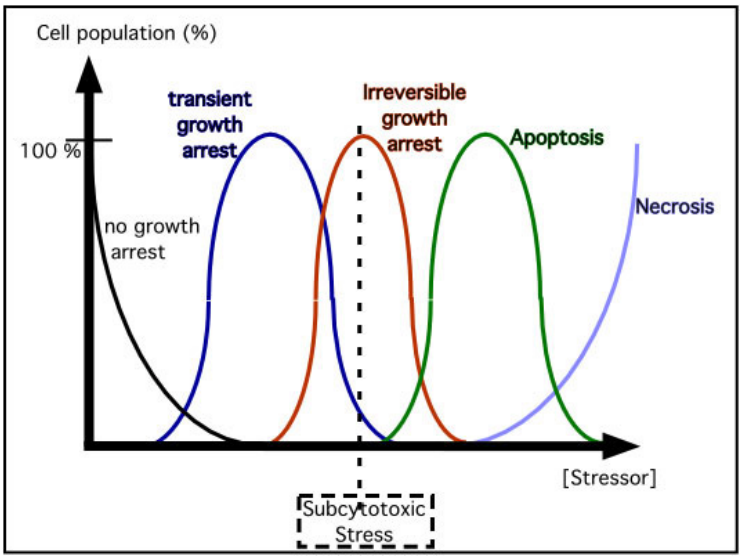

Figure 9: When a cell population of a given cell type is exposed to stress at a subcytotoxic level (vertical dotted line), a gross majority of cells become irreversibly growth arrested and prematurely "senescent" while a minority of other cells might eventually resume mitosis. Taken from Toussaint *et al.*, 2002a.

2000a). Since ROS have been implicated in ageing, oxidative stress may be a useful way to study ageing. Therefore, SIPS can be seen as another model of human ageing mechanisms.

2.3.1. Biomarkers of SIPS induced by oxidative stress

SIPS is similar to RS and represents a state of senescence, as can be observed through several biomarkers. One key biomarker is growth arrest, normally at the G1/S transition. After exposure to H_2O_2, HDFs show a decrease in CPDs (Honda and Matsuo, 1983; Toussaint *et al.*, 1992; Chen and Ames, 1994). Loss of replicative potential has also been shown in HDFs exposed to H_2O_2 or t-BHP (Dumont *et al.*, 2000a).

When HDFs are exposed to repeated stresses of UV, mitomycin C, or strong electromagnetic fields, a few days after the last stress they feature a senescent-like morphology (Rodemann, 1989; Rodemann *et al.*, 1989a; Rodemann *et al.*, 1989b). Exposure to H_2O_2 also induces a senescent-like morphology (Chen and Ames, 1994). Moreover, as previously mentioned, a correlation appears to exist between the changes in morphology and an increase in the proportion of HDFs positive for SA β-gal activity (Toussaint *et al.*, 2000). In fact, an increase in the proportion of SA β-gal positive cells in SIPS has been reported for many types of cells under different stressors, namely in HDFs submitted to oxidative stress (Dumont *et al.*, 2000a; Frippiat *et al.*, 2001; Figure 10).

In SIPS, the common 4,977 bp deletion has been observed in HDFs following t-BHP stress, in the same way it gradually increases with the number of CPDs (Dumont *et al.*, 2000a).

Several genes differentially expressed in RS show similar expression changes in SIPS as a results of oxidative stress by O_2/hyperoxia (Saretzki *et al.*, 1998), H_2O_2 (Chen *et al.*, 1998; Frippiat *et al.*, 2001) or t-BHP (Dumont *et al.*, 2000a).

These biomarkers suggest that the phenotype seen in SIPS represents a form of senescence similar to RS. At the time we began our work, the role of telomeres in SIPS was under debate and will be discussed in chapters 3 and 4.2.

2.4. Relation between ageing, RS, and SIPS

The connection between ageing and RS is not obvious. At least *post partum*, there is no relation between the number of CPDs cells can endure and the age of the donor (Cristofalo *et al.*, 1998b; Figure 11). Chances are previous studies showing otherwise were biased (Cristofalo, 2001). Studies in centenarians failed to find differences in the CPDs cells from centenarians

stressor	cell type	specie	senescent morphology	SA ß-gal	growth arrest	G1 block	reference
tert-butylhydroperoxide	WI-38 fetal lung fibroblasts	human	OK	OK	OK		(Dumont et al., 2000a)
" "	" "	" "	OK	OK	OK		(Dumont et al., 2001)
H_2O_2	IMR-90 fetal lung fibroblasts	" "	OK	OK	OK		(Dumont et al., 2000a)
" "	" "	" "	OK		OK	OK	(Chen et al., 1998)
" "	" "	" "	OK	OK	OK		(Frippiat et al., 2001)
" "	WI-38 fetal lung fibroblasts	" "	OK	OK	OK	OK	(Wolf et al., 2002)
" "	cardiac fibroblasts	rat			OK		(Siwik et al., 2001)
NaCl (hypertonic)	WI-38 fetal lung fibroblasts	human	OK		OK		(Reichelt and Schachtschabel, 2001)
H_2O_2, UV or γ-irradiation	WI-38 and WI-38-hTERT fetal lung fibroblasts	" "	OK	OK	OK		(Gorbunova et al., 2002)
" "	IMR-90 and IMR-90-hTERT fetal lung fibroblasts	" "	OK	OK	OK		(Gorbunova et al., 2002)
" "	HCA2 and HCA2-hTERT fibroblasts	" "	OK	OK	OK		(Gorbunova et al., 2002)
" "	LF1, LF1-hTERT fibroblasts	" "	OK	OK	OK		(Gorbunova et al., 2002)
" "	LF1p21-/-, LF1p21-/-hTERT fibroblasts	" "	OK	OK	OK		(Gorbunova et al., 2002)
UV	HH-8 skin fibroblasts	" "	OK		OK		(Rodemann et al., 1989a)
UVB	melanocytes	" "			OK	OK	(Medrano et al., 1995)
" "	skin fibroblasts	" "	OK	OK	OK		(Chainiaux et al., 2002)
ionizing radiation	aortic endothelial cells	bovine	OK	OK	OK		(Oh et al., 2001)
γ-radiation	several strains of fibroblasts	human			OK	OK	(Di Leonardo et al., 1994)
strong electromagnetic fields	HH-8 (skin) and WI-38 (fetal lung) fibroblasts	" "	OK		OK		(Rodemann et al., 1989b)
ethanol	WI-38 fetal lung fibroblasts	" "	OK	OK	OK		(Dumont et al., 2002)
" "	" "	" "	OK	OK	OK		(Toussaint et al., 1995)
xanthine + xanthine oxidase	cardiac fibroblasts	rat			OK		(Siwik et al., 2001)
mitomycin C	HH-8 fibroblasts	human	OK		OK		(Rodemann, 1989)
bleomycin, actinomycin D	fibroblasts	" "	OK		OK		(Robles and Adami, 1998)
5-bromodeoxyuridine	TIG-7 fibroblasts	" "	OK	OK		OK	(Michishita et al., 1999)
" "	TIG-7 fibroblasts	" "	OK		OK		(Suzuki et al., 2001a)
hydroxyurea	fibroblasts	" "	OK	OK	OK		(Yeo et al., 2000)
taxol	WI-38 and REF-52 fibroblasts	" "	OK			OK	(Trielli et al., 1996)
psoralen photoactivation	skin fibroblasts	" "	OK	OK	OK		(Ma et al., 2002)
hyperoxia	WI-38 fetal lung fibroblasts	" "	OK		OK	OK	(von Zglinicki et al., 1995)
" "	BJ and MRC-5 fibroblasts	" "	OK		OK		(Saretzki et al., 1998)
" "	WI-38 and TIG-1 fibroblasts	" "	OK		OK		(Honda and Matsuo, 1983)
homocysteine	umbilical vein endothelial cells	" "		OK	OK		(Xu et al., 2000)
IL-1α or TNF-α	WI-38 fetal lung fibroblasts	" "	OK	OK	OK		(Dumont et al., 2000b)
TGF-ß1	IMR-90 fetal lung fibroblasts	" "	OK	OK			(Frippiat et al., 2001)

Table 3: Non-exhaustive list of the different models of SIPS in normal cell types together with the different biomarkers detected in these models: senescent morphology, SA β-gal activity, growth arrest, and G1 block. Adapted from Dierick *et al.*, 2003.

could endure (Tesco *et al.*, 1998). In addition, they raised doubts on whether telomere shortening occurs *in vivo* and whether senescence-associated genes *in vitro* are also differentially expressed *in vivo* (Mondello *et al.*, 1999). In fact, gene expression patterns show differences between *in vitro* senescent cells and cells from old donors (Takeda *et al.*, 1992). Recently, the relation between SA β-gal and *in vivo* ageing has also been attacked (Going *et al.*, 2002).

It is known that cells from older donors have a slower proliferative capacity (Waters and Walford, 1970; Hayflick, 1994). This effect, known as the latent period, occurs because fewer cells are in the replication cycle, not because they take longer to divide (Ponten *et al.*, 1983; Karatza *et al.*, 1984). Therefore, changes occur with age at a cellular level. In some tissues, such as the immune system, decreased proliferative ability may play a role in age-related degeneration (reviewed in Effros, 1996). Nevertheless, RS does not appear to be a faithful model of changes occurring *in vivo* (Gershon and Gershon, 2000b for arguments).

As mentioned before, a relation exists between stress resistance and ageing. Whether SIPS is a precise simulation of *in vivo* degeneration of proliferative tissues remains a point of discord. It appears likely, however, that the phenotype of SIPS is not exactly RS (Wright and Shay, 2001; Toussaint *et al.*, 2002a). For example, one key difference between RS and SIPS is telomere shortening, which undeniably occurs in RS but whose involvement in SIPS is under discussion, as will be discussed in chapter 4.2. Yet *in vivo* cell senescence can be found without telomere shortening (Melk *et al.*, 2003), suggesting that RS may not be the prevailing mechanism *in vivo*. Since cells taken from old donors do not endure less CPDs, one hypothesis is that *in vivo* RS does not widely occur and that cellular senescence *in vivo* is dependent on stress factors. When cell lines are derived from people, the selected cells are those that grow because people, even very old people, never run out of proliferating cells (Tesco *et al.*, 1998; Cristofalo, 2001). In fact, from a simple mathematical perspective, it appears unlikely that RS occurs *in vivo*. Assuming HDFs endure 50 CPDs, 2^{50} is more than enough cells for several lifetimes (Hayflick, 1994). Therefore, only a minority of cells may reach RS *in vivo* (Toussaint *et al.*, 2002b). Of course that even a small percentage of senescent cells may interfere with tissue homeostasis and function (Shay and Wright, 2000), but RS such as defined by the phase III phenomenon appears unlikely to be widely present *in vivo*.

Although the relation between a species' longevity and the CPDs its cells can endure *in vitro* exists, it is also debatable if this is related to ageing since optimal culture conditions vary from species to species. As mentioned in chapter 2.3., O_2 partial pressure can affect cellular proliferation and recent results show that O_2 limits the replicative capacity of murine fibroblasts (Parrinello *et al.*, 2003). These results show that comparisons between different species, such as

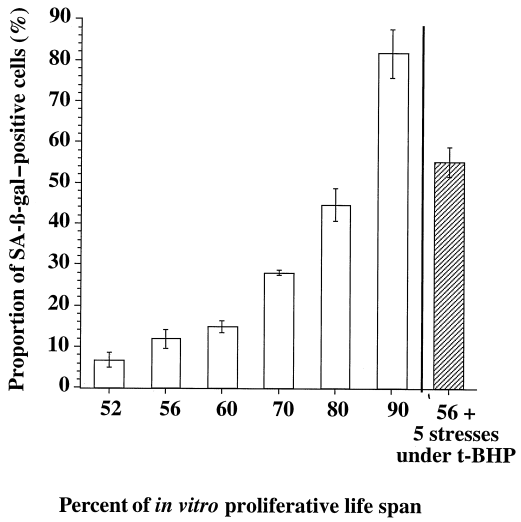

Figure 10: Effects of repeated t-BHP stresses on the proportion of cells positive for SA β-gal activity. WI-38 HDFs from 52 to 90% of their proliferative lifespan (white columns) and HDFs at 56% of their proliferative lifespan submitted to five stresses of 1 h with 30 µM t-BHP (dashed column). Taken from Dumont *et al.*, 2000a.

those described in chapter 2.1., may be biased due to intra-species differences in O_2 sensitivity; instead of showing maximum cellular proliferate capacity, these results show O_2 sensitivity (Toussaint *et al.*, 2002b). In addition, due to the positive correlation between body size and longevity (Promislow, 1993), perhaps cells taken from long-lived animals endure more CPDs because of the difference in size, not due to the difference in longevity.

Senescent cells and senescence-associated biomarkers can be found *in vivo* (Paradis *et al.*, 2001; Going *et al.*, 2002). Interestingly, stress-prone tissues appear to be the most affected. HDFs cultured from distal lower extremities of patients with venous reflux, which precedes the development of venous ulcers, display characteristics of senescent cells (Mendez *et al.*, 1998a; Mendez *et al.*, 1998b). Similar results also relate cellular senescence to atherosclerosis (Minamino *et al.*, 2002) and benign prostatic hyperplasia, a common age-related male pathology (Castro *et al.*, 2003). Senescence and inflammatory processes may also be related in age-related pathologies such as osteoarthritis (Martin and Buckwalter, 2002; Price *et al.*, 2002) and skin ageing (Giacomoni *et al.*, 2000). Indeed, repeated stimulation of WI-38 HDFs with pro-inflammatory cytokines interleukin-1a or tumour necrosis factor-α induces SIPS (Toussaint *et al.*, 1996). These cytokines' circulating levels increase *in vivo* (reviewed in Lio *et al.*, 2003), favouring inflammation and SIPS, so *in vivo* senescence may be a result of SIPS rather than RS. Some data indicate that chronic stressors may accelerate risk of a host of age-related diseases by prematurely ageing the immune response (Kiecolt-Glaser *et al.*, 2003). Lastly, as hinted by the above mentioned results on the impact of O_2 in cell proliferation, RS for many cell lines *in vitro* and *in vivo* may instead be better defined as SIPS resulting from oxidative stress.

The reasons why we employed SIPS in this study are: 1) we work with human cells, not that of animal models. 2) RS does not appear to be a faithful example of human ageing while SIPS, though not being a perfect model of human ageing, can occur *in vivo* and be related to both organismal ageing and cancer (Serrano and Blasco, 2001). 3) SIPS is involved in several pathologies and the elucidation of the mechanisms responsible for SIPS can have medical applications.

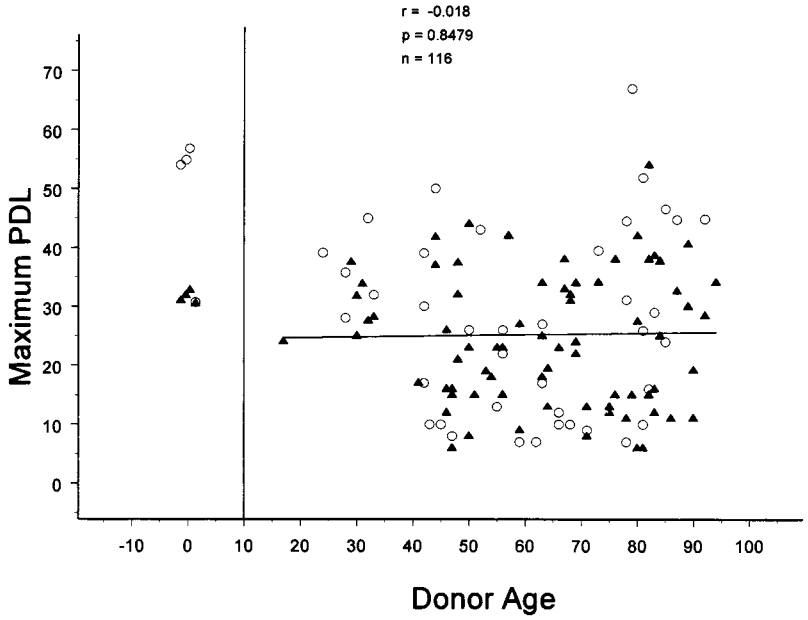

Figure 11: Relationship between in vitro proliferative capacity of postnatal skin fibroblast cell lines and donor age (in years). A regression line (r = -0.018, P = 0.85, and n = 116) is shown. Males are represented by closed triangles and females by open circles. Taken from Cristofalo *et al.*, 1998b.

CHAPTER 3: CELL CYCLE REGULATION BY THE TELOMERES

3.1. Telomere shortening and RS

Early studies by Hermann Muller and Barbara McClintock showed that the ends of chromosomes are capped by a structure called the telomere to prevent chromosome fusions (Muller, 1938; McClintock, 1941). In the 1970's, as the mechanisms behind DNA replication were becoming better understood, it became clear that DNA polymerase, the enzyme responsible for DNA replication, could not fully synthesize the 3' end of linear DNA. In 1972, James Watson called this the end-replication problem (Watson, 1972; Figure 12). At about the same time, in a Moscow subway station, Alexey Olovnikov also recognized Watson's problem in an analogy between the track that represented the DNA and the train that represented DNA polymerase. Yet Olovnikov went further to propose that the end-replication problem would result in telomere shortening with each round of replication and that this mechanism could be the cause of RS (Olovnikov, 1971, 1973). Soon after, studies by Leonard Hayflick and colleagues found that the nucleus controls RS (Wright and Hayflick, 1975; Muggleton-Harris and Hayflick, 1976).

Olovkikov's model turned out to be incredibly accurate. Telomere shortening is now considered the main causal mechanism of RS and telomere length is the molecular clock that counts the CPDs cells endure (reviewed in Wright and Shay, 2001). Although it was previously known that telomere shortening occurs in each subcultivation (Harley *et al.*, 1990), the key finding relating the telomeres to RS was made in 1998 by scientists at Geron Corporation. Telomerase is a reverse-transcriptase enzyme that elongates the telomeres (Greider and Blackburn, 1985) thus counteracting the normal telomere erosion. Telomerase has two components: an RNA component (Feng *et al.*, 1995) and a catalytic subunit (Lendvay *et al.*, 1996; Lingner *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997; Figure 13). Previously, it was already apparent an association between telomere shortening, telomerase, and immortality in tumour cell lines (Counter *et al.*, 1992). Yet the definitive breakthrough was that expression of the catalytic subunit of human telomerase (hTERT) in both retinal pigment epithelial cells and foreskin fibroblasts avoids RS (Bodnar *et al.*, 1998). HDFs immortalized with hTERT divide vigorously, do not show increased staining for SA β-gal, and do not show signs of transformation (Jiang *et al.*, 1999; Morales *et al.*, 1999). Even expression of hTERT in old HDFs appears to reverse the loss of function characteristic of senescent cells (Funk *et al.*, 2000). It

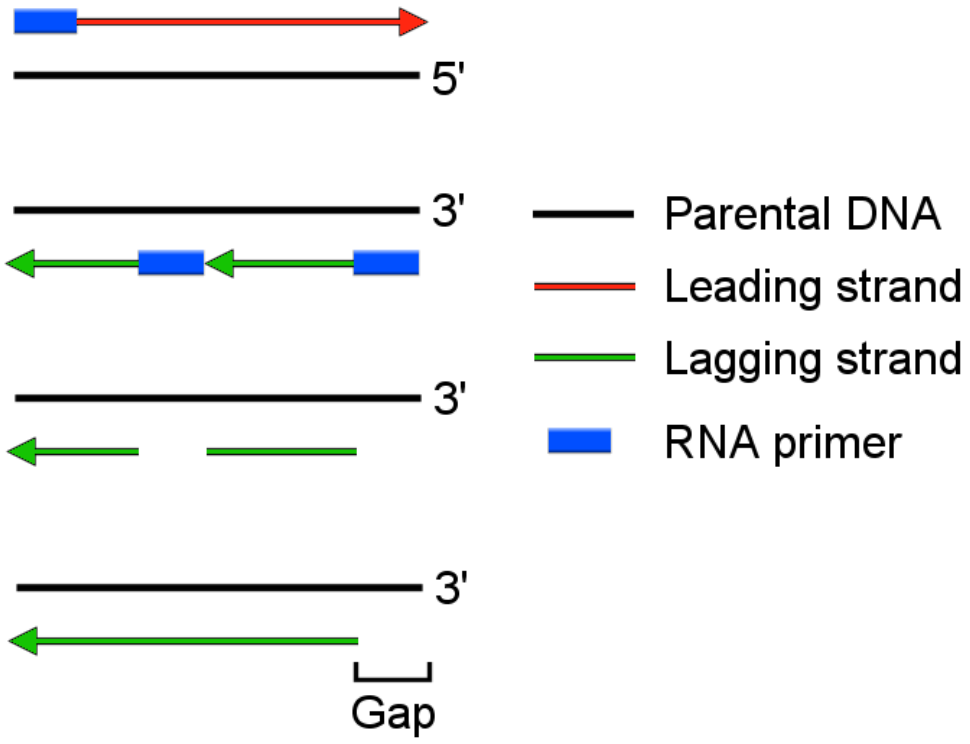

Figure 12: DNA polymerase requires an RNA primer to initiate synthesis in the 5'→3' direction. At the end of a linear chromosome, DNA polymerase can synthesize the leading strand until the end of the chromosome. In the lagging strand, however, DNA polymerase's synthesis is based on a series of fragments, called Okazaki, each requiring an RNA primer. Without DNA to serve as template for a new primer, the replication machinery is unable to synthesize the sequence complementary to the final primal event. The result is the "end-replication problem" in which sequence is lost at each round of DNA replication. Adapted from Wynford-Thomas and Kipling, 1997.

appears that ectopic hTERT expression is sufficient to restore telomerase activity in human cells (Counter *et al.*, 1998).

Transient expression of hTERT for 7 CPDs in HDFs elongated the shortest telomeres by 2.5 kilobase pairs (kbp). Afterwards, these HDFs divided for roughly 50 CPDs with a telomeres shortening of 50 bp per division before reaching RS. These results strongly argued that telomere length, not hTERT expression, is the key in avoiding RS (Steinert *et al.*, 2000) and established telomere length as the clock that keeps track of CPDs and originates RS. In addition, recent results show that the senescent cells in dividing cultures have shorter mean telomere lengths than dividing cells of the same population (von Zglinicki *et al.*, 2003; Martin-Ruiz *et al.*, unpublished).

Telomerase is not the only mechanism capable of elongating the telomeres. There are several immortal telomerase-negative cell lines with, typically, a great variety of telomere lengths (Bryan *et al.*, 1995; Bryan *et al.*, 1997). Although the exact mechanisms behind what is called alternative lengthening of telomeres remain unknown, recombinational processes may be involved (McEachern and Blackburn, 1996; Dunham *et al.*, 2000).

Either using telomerase or not, all known immortal cell lines must stabilize their telomeres (reviewed in Colgin and Reddel, 1999; Stewart and Weinberg, 2000). Tumour development, in particular, is dependent on telomere stabilization, normally by telomerase (Chen *et al.*, 2000a). In contrast, telomerase inhibition can induce senescence in cancer cells (Shammas *et al.*, 1999). The role of telomeres in tumorogenesis will be further discussed in chapter 3.3. Lastly, unicellular eukaryotes must also stabilize their telomeres. Indeed, defects in telomere replication have been shown to trigger senescence in yeast (Lundblad and Szostak, 1989) and in the protozoan Tetrahymena (Yu *et al.*, 1990).

3.2. How telomere dysfunction induces senescence

Although telomere length regulates RS and can be seen as a mitotic clock, it is neither the only nor the ultimate timekeeper of cells (reviewed in Blackburn, 2000). During telomerase-immortalization of human cell lines, including HDFs, several researchers noticed that immortalized cells had shorter telomeres than growth arrested controls (Ducray *et al.*, 1999; Yang *et al.*, 1999; Zhu *et al.*, 1999). Surprisingly, immortalized cells featured less chromosome fusions, which are the most noticeable outcome of short telomeres (Hande *et al.*, 1999). Similarly, it was noticed in yeasts that certain telomerase-negative strains would senesce with longer telomeres than immortal telomerase-positive strains (Prescott and Blackburn, 1997; Roy

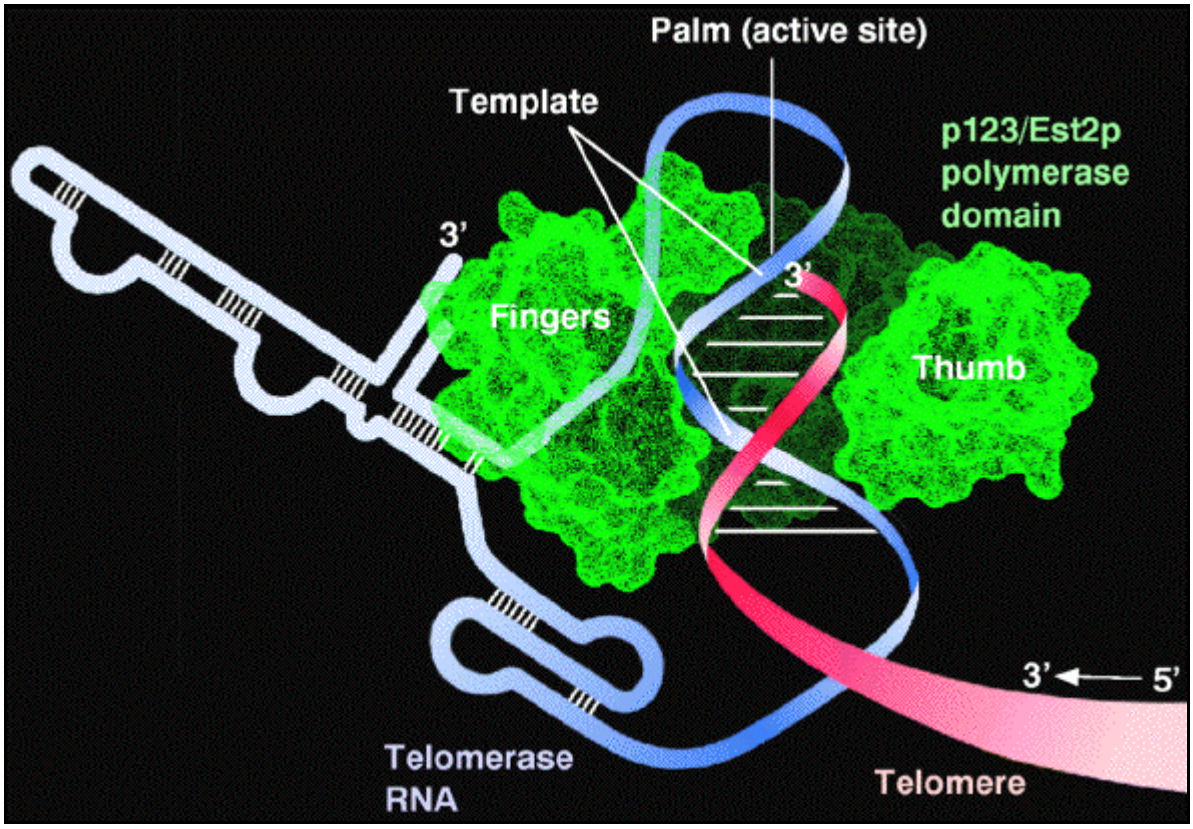

Figure 13A: Model of telomerase as an RNA-reverse transcriptase complex. The p123/Est2p subunit (green) is based on the right hand model of HIV-1 reverse transcriptase; thumb and fingers extend toward the reader. The reverse transcriptase motifs are in the palm, and the active site aspartates are near the 3' end of the telomeric DNA substrate (red). The RNA subunit (blue) has its template region in the palm; the location of the remainder of the RNA is unknown and is shown schematically in its secondary structure representation. The telomeric DNA substrate is shown base-paired but not intertwined with the RNA subunit. The extent of base pairing and the sites of interaction of the nucleic acids with the protein are not known. Taken from Lingner *et al.*, 1997.

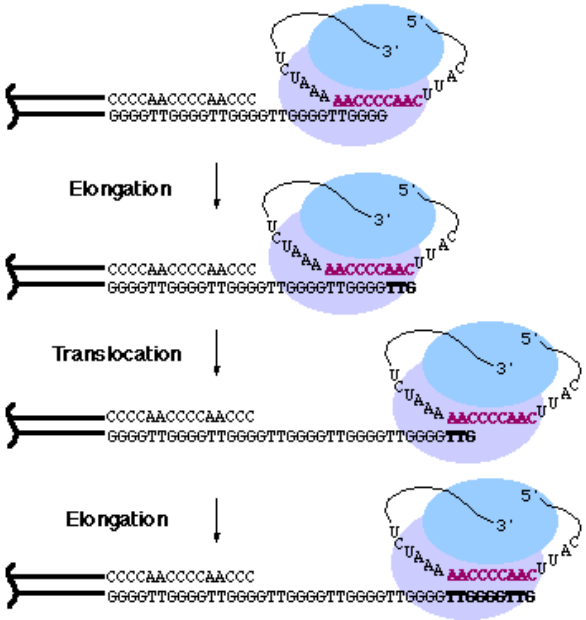

Figure 13B: Telomerase, a reverse transcriptase with an RNA subunit, extends the lagging DNA strand. Taken from TelDB at Washington University School of Medicine based on the work of Carol Greider: http://www.genlink.wustl.edu/teldb/

et al., 1998). More recently, studies with HDFs strains derived from different individuals showed great variability in telomere length at the time of senescence (Serra and von Zglinicki, 2002). Since telomere length alone could not explain these observations, other players had to be involved.

Using electron microscopy, it was revealed that telomeres are not linear, but instead appear to form duplex loops, called t-loops. Crucial in these loops are the telomeric repeat-binding factors TRF1 (van Steensel and de Lange, 1997) and TRF2 (Smogorzewska *et al.*, 2000). In particular, TRF2 can remodel linear telomeric DNA into t-loops (Griffith *et al.*, 1999). Although much remains unknown, the prevailing hypothesis is that these loops stabilize or cap the telomeres (Figure 14). Capping may protect the telomeres from being recognized as DNA damage. TRF2 protects telomeres (van Steensel *et al.*, 1998): inhibition of TRF2 induces apoptotic cell death (Karlseder *et al.*, 1999) while overexpression of TRF2 reduces the senescent checkpoint of cells in terms of telomere length (Karlseder *et al.*, 2002). These results suggested that telomere capping, not just telomere length, is crucial in avoiding telomere dysfunction and preventing apoptosis and senescence. Recent results showing that telomerase disruption can slow cell proliferation and alter the 3' single-stranded telomeric overhang without telomere shortening support this view (Masutomi *et al.*, 2003). One plausible hypothesis is that telomere shortening may destabilize or even prevent the capping of telomeres, leading to senescence (Shay, 1999). Importantly, we still lack a detailed view of the mechanisms involved. For instance, murine TRF1 knock-out mice die at embryonic stages suggesting that TRF1 has an essential function that is independent of telomere length regulation (Karlseder *et al.*, 2003).

Whether the end-replication problem alone is responsible for telomere shortening has become a centre for debate. Senescence can occur in HDFs in the absence of cell division and short telomeres. HDFs kept confluent for long periods of time--up to 12 weeks--exited the cell cycle. The small proportion of HDFs that continued dividing endured fewer cumulative population doublings than normal presumably due to compensatory cycling (Munro *et al.*, 2001). Although quiescent cells do not appear to lose telomeres (von Zglinicki, 1998 & 2000), HDFs endure an accelerated telomere shortening following extensive periods of confluency (Sitte *et al.*, 1998). One hypothesis is that telomere dysfunction occurs in confluent cells despite lack of telomere shortening. Therefore, the end-replication problem as a model to explain telomere shortening may be biased. Telomeres end in a single-stranded G-rich 3'-overhang, presumably as a result of C-rich strand degradation during telomere processing (Wellinger *et al.*, 1996; Makarov *et al.*, 1997; Wright *et al.*, 1997). Recent results suggest that erosion of the overhang occurs at senescence, prevented by telomerase expression. Progressive erosion appears to be a

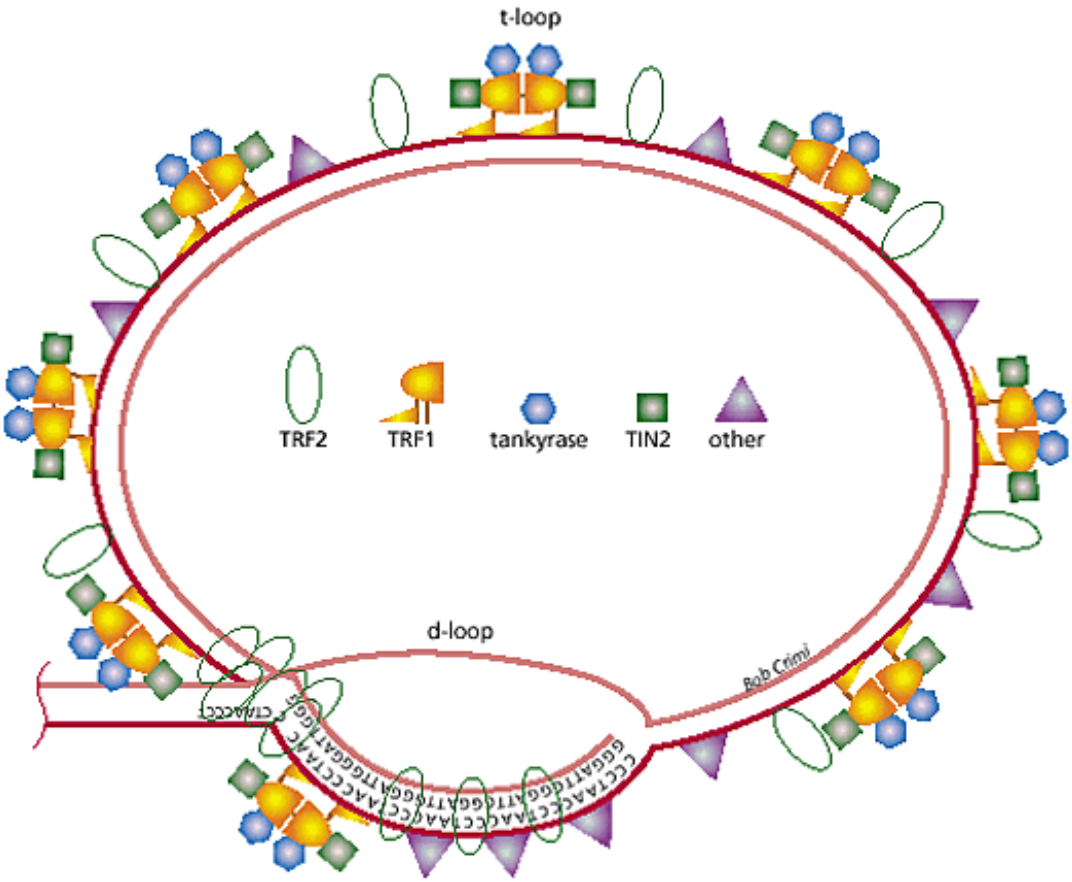

Figure 14: A new view of the mammalian telomere. At the end of the chromosome, the mammalian double-stranded telomere bends back on itself forming a large telomere loop (t-loop). The 3' G-rich single-stranded overhang at the end of the t-loop invades the double-stranded telomere and produces a displacement loop (d-loop). The sequestration of the G-rich single-stranded overhang may prevent inappropriate DNA damage checkpoint responses. The telomere-specific DNA-binding proteins TRF1 and TRF2 are required for normal telomere function along with the TRF1-associated proteins tankyrase and TIN2. Homodimerization of TRF1 is required to form a stable complex with DNA in mammalian cells. Whereas tankyrase may only associate with TRF1 transiently, TIN2 appears to be recruited to TRF1, and this complex, together with TRF2, may make the 3' G-rich overhang inaccessible to telomerase. Thus, TRF1 and its associated proteins (TIN2 and tankyrase), TRF2, the telomerase holoenzyme, and perhaps as yet unidentified proteins, are important regulators of human telomere length. Taken from Shay, 1999.

result of cell division and not an effect of senescence (Stewart *et al.*, 2003). As such, the exact molecular mechanisms behind telomere shortening and dysfunction remain undetermined.

Another source of controversy is whether it is mean telomere length or the shortest telomere to trigger RS. Evidence from mice indicates that the shortest telomeres, not mean telomere length, are responsible for inducing RS (Hemann *et al.*, 2001). Yet in HDFs the onset of RS shows a better correlation with mean telomere length than with chromosomes with the shortest telomere (Martens *et al.*, 2000). Therefore, the debate rages on.

3.2.1. Uncapped telomeres recognized as DNA damage

Even before hTERT-derived immortalization, it was possible to immortalize HDFs using viral genes such as the simian virus 40 (SV40) T-antigen, E1A and E1B from adenovirus, or the human papillomavirus E6 and E7 genes. The E1B and E6 proteins bind and inactivate the tumour suppressor protein p53 while E1A and E7 bind and inactivate the retinoblastoma protein, also called pRb (Dyson *et al.*, 1989; Werness *et al.*, 1990). Immortalization requires E6 and E7 or E1A plus E1B, so both p53 and pRb must be inactive (Figure 15). SV40 immortalization is also dependent on inactivation of both p53 and pRb (Shay *et al.*, 1991). These findings led to the present concept that two pathways are responsible for inducing senescence. Confirming these suspicions, inhibition of p53 and pRb by antisense technology caused cells to endure 50 CPDs more than normal (Hara *et al.*, 1991).

Re-expression of pRb in pRb/p53-defective immortal tumour cells induces senescence (Xu *et al.*, 1997). In agreement with its anti-oncogenic profile, pRb is a central regulator of cell cycle progression and its state of phosphorylation determines cell cycle regulation (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; reviewed in Herwig and Strauss, 1997). Hyperphosphorylated pRb allows the cell cycle to proceed while hypophosphorylated pRb prevents cell cycle progression. Presumably, pRb operates through inactivation of the E2F family of transcription factors, responsible for transcription of several genes involved in G1/S transition and DNA synthesis (Weintraub *et al.*, 1992; Farnham *et al.*, 1993; Sladek, 1997; Campisi, 1999). Briefly, the phosphorylation of pRb is dependent on cyclin-dependent kinases (CDKs) that govern the progression through the various phases of the cell cycle (reviewed in Lees, 1995). Inactivation of the G1 CDKs, responsible for the phosphorylation of pRb, prevents transition from phase G1 to phase S and blocks the cell cycle, originating, for example, RS.

Cyclin-dependent kinase inhibitors (CDKIs), as the name implies, inhibit the activity of CDKs. One of such proteins is $p16^{INK4a}$, whose lack of activity was found in cancers, correlating

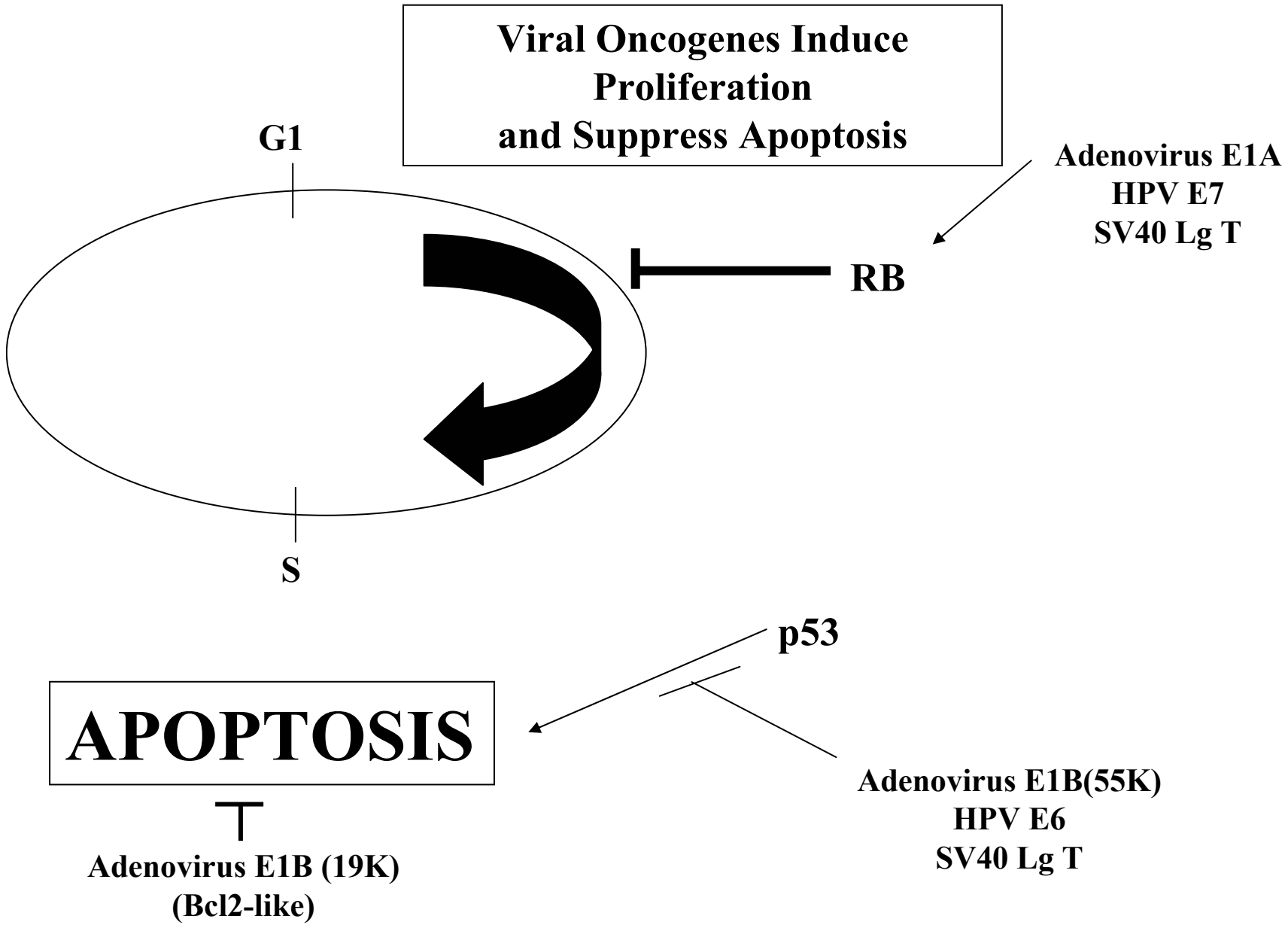

Figure 15: Immortalization through viral proteins. Adapted from Amy Yee, Tufts University School of Medicine: http://www.tufts.edu/med/biochemistry/faculty/yee/yee.html

with pRb but not p53 (Okamoto *et al.*, 1994; Whitaker *et al.*, 1995). $p16^{INK4a}$ disrupts and inhibits the activities of CDK4 and CDK6, thus preventing cell cycle progression (Serrano *et al.*, 1993; Hara *et al.*, 1996). Exogenous expression of $p16^{INK4a}$ induces senescence in young HDFs and in immortal cells without p53 activity (McConnell *et al.*, 1998; Vogt *et al.*, 1998). In addition, immortalization may also be achieved by disruption of both $p16^{INK4a}$ and p53 (Rogan *et al.*, 1995). pRb is needed for growth suppression mediated by $p16^{INK4a}$ (Medema *et al.*, 1995). These results suggest that $p16^{INK4a}$ acts upstream of pRb in regulating RS. If telomere dysfunction activates $p16^{INK4a}$, the mechanism is not known despite $p16^{INK4a}$'s involvement, in parallel with p53, in the DNA damage response (Shapiro *et al.*, 1998), as will be detailed in chapter 4. Furthermore, the levels of $p16^{INK4a}$ increase in senescent, but not pre-senescent, HDFs (Wong and Riabowol, 1996), suggesting other signalling pathways originating in the telomeres trigger RS (Alcorta *et al.*, 1996).

Another important CDKI is $p21^{WAF1}$, which also has the ability to block the cell cycle by inhibiting CDK2, CDK4, and CDK6 and thus preventing pRb phosphorylation (Harper *et al.*, 1993; Noda *et al.*, 1994; Harper *et al.*, 1995; Stein *et al.*, 1999). Other mechanisms have been proposed, such as disruption of DNA synthesis by binding and inhibiting a protein called proliferating cell nuclear antigen (Waga *et al.*, 1994; Chen *et al.*, 1995b; Luo *et al.*, 1995). Either way, $p21^{WAF1}$ can induce senescence independently of $p16^{INK4a}$ (McConnell *et al.*, 1998; Vogt *et al.*, 1998). Since $p21^{WAF1}$ expression levels increase in pre-senescent cells--i.e. before $p16^{INK4a}$ overexpression--, $p21^{WAF1}$ is likely to trigger senescence before $p16^{INK4a}$ (Tahara *et al.*, 1995; Alcorta *et al.*, 1996; Wong and Riabowol, 1996; Dulic *et al.*, 2000). In contrast, $p16^{INK4a}$ remains overexpressed in senescent cells while $p21^{WAF1}$ levels wane (Stein and Dulic, 1998).

p53 induces $p21^{WAF1}$ (el-Deiry *et al.*, 1993). Microinjection of antibodies directed against p53 into senescent HDFs prevented $p21^{WAF1}$ expression and resulted in cells entering the S phase and proliferating (Bond *et al.*, 1996). Although it is possible that other downstream targets of p53 exist, $p21^{WAF1}$ is involved in RS induced by p53 (reviewed in Wang *et al.*, 2003).

In brief, p53 is a multifunctional protein, a transcription factor capable of acting both as a transcriptional activator and suppressor. Overexpression of p53 leads to cell cycle arrest or apoptosis (Sugrue *et al.*, 1997). The induction of p53 by DNA-damaging agents led to the suggestion that p53 is a checkpoint factor that prevents cells from accumulating mutations by inducing apoptosis or growth arrest (reviewed in Ko and Prives, 1996). p53 may help maintain genetic stability (Linke *et al.*, 2003 for arguments).

Increased levels of p53 have been associated with critically short telomeres (Vaziri and Benchimol, 1996; Gonzalez-Suarez *et al.*, 2000), p53-deficiency attenuated the phenotype of

telomere dysfunction (Bond *et al.*, 1994; Bond *et al.*, 1996; Chin *et al.*, 1999), and microinjection of anti-p53 antibodies restores cell division in senescent HDFs (Gire and Wynford-Thomas, 1998). Thus p53 is probably responsible for recognizing dysfunctional telomeres--e.g. critically short telomeres--as DNA damage and triggering RS. Indeed, activation of p53 occurs as HDFs approach senescence (Atadja *et al.*, 1995; Kulju and Lehmn, 1995; Bond *et al.*, 1996). Therefore, p53 appears to be the major initiator of senescence, while $p16^{INK4a}$ presumably maintains senescence (Alcorta *et al.*, 1996; Dulic *et al.*, 2000; Serrano and Blasco, 2001; Wang *et al.*, 2003).

$p16^{INK4a}$ appears to be important in the response to DNA damage (Robles and Adami, 1998; Shapiro *et al.*, 1998; Schmitt *et al.*, 2002; te Poele *et al.*, 2002) and other stimuli like oncogenic signals (Serrano *et al.*, 1997). The immortalization of human epithelial cells requires inactivation of $p16^{INK4a}$--or E7 expression to inhibit pRb--in addition to hTERT activity (Kiyono *et al.*, 1998). Yet under 2% O_2, epithelial cells can be immortalized with hTERT activity alone, suggesting that stressful culture conditions may activate $p16^{INK4a}$ and induce senescence independently of the telomeres (Ramirez *et al.*, 2001; Rheinwald *et al.*, 2002). The role of $p16^{INK4a}$ in the DNA damage response will be further discussed in chapter 4. Lastly, $p16^{INK4a}$ does not appear to be involved in RS of murine cells (Smogorzewska and de Lange, 2002), indicating that the regulation of RS and telomere dysfunction in murine and human cell lines is different, as suggested by others (Hamad *et al.*, 2002; Kim *et al.*, 2002).

p53 itself may be upregulated. Although the issue is still controversial, some evidence indicates that the ATM gene, or other players involved in DNA damage response, may be the "sensor" that detects telomere dysfunction and then regulates p53 (Vaziri *et al.*, 1997; Rouse and Jackson, 2002). Moreover, recent results confirm that the chromosome ends of senescent cells directly contribute to the DNA damage response and that uncapped telomeres directly associate with many DNA damage response proteins (d'Adda di Fagagna *et al.*, 2003).

Other players are likely to be involved. For example, the INKa locus also encodes $p14^{ARF}$ (Mao *et al.*, 1995; Stone *et al.*, 1995), a tumour suppressor protein mutated in several human tumours (Eischen *et al.*, 1999; Lindstrom *et al.*, 2001). In mice, $p19^{ARF}$ can induce growth arrest by interacting with MDM2 (Stott *et al.*, 1998), a protein that degrades p53 (Momand *et al.*, 1992), or through p53-independent pathways (Korgaonkar *et al.*, 2002). In general, $p16^{INK4a}$ appears to have a more important role in SIPS and RS of HDFs than ARF while mouse fibroblasts appear to rely more on ARF, and so we focus on $p16^{INK4a}$ (reviewed in Serrano and Blasco, 2001; Collins and Sedivy, 2003). Intriguingly, some evidence suggests that the Bmi-1 protein, which controls gene expression through methylation, represses the INK4a locus and

extends replicative life span (Itahana *et al.*, 2003). Another possible player is the cyclin D1 oncogene, which associates with CDKs 4 and 6 (Matsushime *et al.*, 1992; Meyerson and Harlow, 1994). Ectopic overexpression of cyclin D1 inhibits DNA synthesis and cell growth (Pagano *et al.*, 1994; Atadja *et al.*, 1995; Han *et al.*, 1995). Recent results suggest that a novel transcriptional element regulates cyclin D1, and possibly other senescence-associated genes, in senescence cells (Berardi *et al.*, 2003).

Immortalization with viral proteins is not as simple as it may seem at first. Infection of HDFs with viral oncogenes results in an extended life span after which cells enter a stage called crisis (reviewed in Goldstein, 1990; McCormick and Campisi, 1991; Wei and Sedivy, 1999). For example, introduction of SV40 sequences confers an extra 20-30 CPDs to pre-senescent HDFs. During crisis, cells proliferate but the rate of apoptotic cells gradually increases and thus cell numbers eventually diminish (Macera-Bloch *et al.*, 2002). Since both p53 and pRb/$p16^{INK4a}$ pathways are inactive and chromosomal instability and fusions are abundant, crisis is thought to emerge due to extremely short telomeres. Occasionally, immortal cells emerge from crisis with stabilized telomeres, normally involving telomerase activation (reviewed in Stewart and Weinberg, 2000; Mathon and Lloyd, 2001). In a sense, crisis can be seen as the ultimate consequence of telomere dysfunction (Figure 16).

Even assuming that the p53 and pRb/$p16^{INK4a}$ pathways explain RS, they do not entirely explain the gradual ageing of cells in culture. One hypothesis is that telomeres exert a position effect on nearby genes. Telomere position effect was first identified in yeast (Gottschling *et al.*, 1990) but its role in the cellular ageing of human cells has been defended (Baur *et al.*, 2001). Perhaps telomere shortening causes the underexpression of important sub-telomeric genes that contribute to the changes seen in the *in vitro* ageing of human cells. Another alternative is that cell populations become more heterogeneous as they age. For example, since the percentage of cells actively dividing decreases with CPD, it is normal that the cell population as a whole ages, without changes other than more cells entering RS.

In conclusion, whatever changes occur during telomere dysfunction, the mechanisms triggering growth arrest appear to involve DNA damage pathways. As such, the most likely explanation is that dysfunctional telomeres are recognized as DNA damage and repairing the short telomeres leads to chromosome fusions. Although unidentified genes may also be involved (Sugawara *et al.*, 1990; Ogata *et al.*, 1993; Blasco and Hahn, 2003; Yawata *et al.*, 2003), the most widely accepted hypothesis is that the p53 and pRb/$p16^{INK4a}$ pathways collaborate to stop cellular proliferation derived from telomere shortening in normal HDFs. Probably, the p53 pathway involving $p21^{WAF1}$ is activated beforehand, while $p16^{INK4a}$ prevails under strong

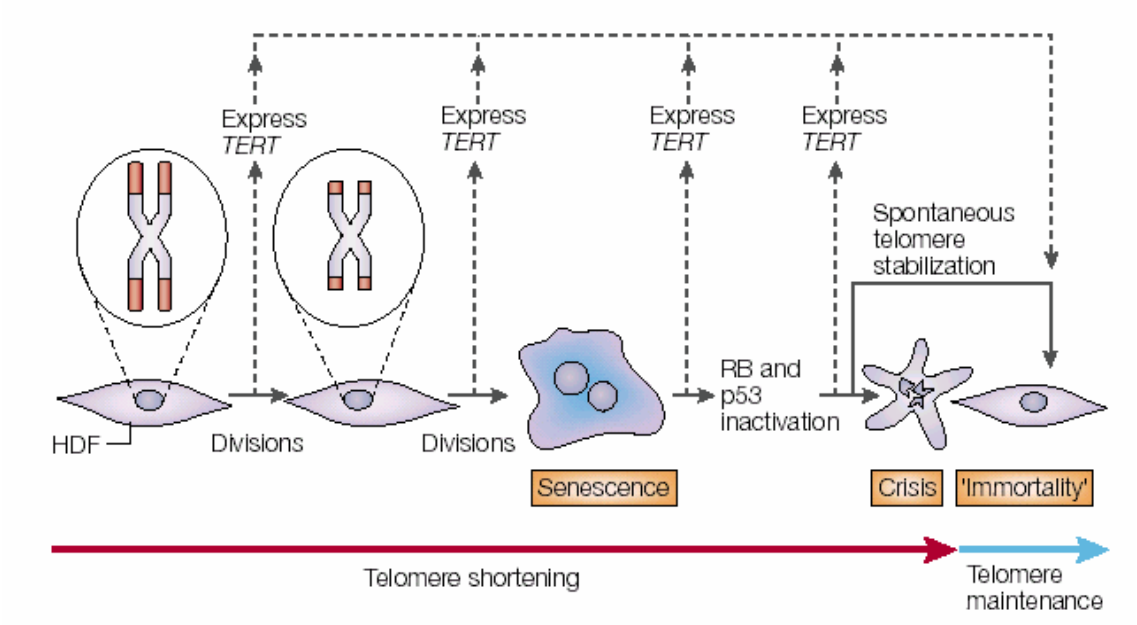

Figure 16: Telomere shortening determines the proliferative lifespan of human diploid fibroblasts. After 60–80 population doublings in culture, short telomeres in human diploid fibroblasts trigger a damage response that leads to senescence. Overcoming senescence, by abrogation of the pRb and p53 tumour-suppressor pathways, allows continued division until critically short telomeres trigger crisis. Rare cells emerge from crisis by activating mechanisms for telomere stabilization. Exogenous expression of telomerase reverse transcriptase (TERT) at any stage in the replicative history allows immortalization of these cells. Taken from Mathon and Lloyd, 2001.

physiological stimuli or stress and to maintain cells growth arrested, a state also called quiescence.

3.3. Ageing, cancer, and the telomeres

Telomere shortening is most likely a tumour suppressor mechanism. Mice lacking telomerase were viable up to six generations. Telomeres gradually shortened and cells from animals of generation four displayed aneuploidy and other chromosomal aberrations. Knocking-out telomerase in mice through deletion of the RNA component from the germline, while not preventing cancer (Blasco *et al.*, 1997; Rudolph *et al.*, 1999), appears to increase cancer resistance (Gonzalez-Suarez *et al.*, 2000; Rudolph *et al.*, 2001). On the other hand, telomerase overexpression in mice promoted cancer development (Gonzalez-Suarez *et al.*, 2001; Artandi *et al.*, 2002). In addition, as presented in chapter 3.2.1, the connection between the telomere signalling pathways and cancer is obvious (reviewed in Fearon, 1997). The human Li-Fraumeni syndrome has been associated with mutations in p53 and is characterized by increased cancer incidence (reviewed in Varley *et al.*, 1997). Human germline mutations in p53 are also associated with a major cancer risk (Hwang *et al.*, 2003) and p53 may suppress telomerase (Stampfer *et al.*, 2003). Retinoblastoma is also recognized as hereditary cancer (Murphree and Benedict, 1984; Weichselbaum *et al.*, 1988; Goodrich and Lee, 1993). Germline mutations in $p16^{INK4a}$ have too been implicated in familial melanoma (Hussussian *et al.*, 1994). Lastly, telomerase activation has been associated with skin malignancy as a result of exposure to UV (Ueda *et al.*, 1997).

More dubious is the role of telomeres in animal ageing. Telomerase expression has been found in lobsters and trout, two species in which ageing remains undetected (Klapper *et al.*, 1998a & 1998b). On the other hand, in the frog *Xenopus laevis*, another animal with a slow rate of ageing (Brocas and Verzar, 1961), not only a great variation in telomere length exists (Bassham *et al.*, 1998) but telomere length can diminish from parents to offspring, despite telomerase activity in germ cells, with no detectable consequences (Mantell and Greider, 1994). Recently, telomerase expression has also been found in zebrafish, though little is know about its ageing process (Kishi *et al.*, 2003). Lastly, telomeric DNA in chicken appears to be ten times bigger than in humans (Delany *et al.*, 2003) and chicken somatic tissues express telomerase (Venkatesan and Price, 1998), showing how our knowledge of telomere biology is limited regarding other species.

No connection exists between mean telomere length and mammalian ageing. Of all studied primates, humans appear to have the shortest telomeres and the longest lifespan (Kakuo *et al.*, 1999; Steinert *et al.*, 2002). As mentioned before, telomerase overexpression does not affect ageing in mice (Gonzalez-Suarez *et al.*, 2001). Mice also have long telomeres and feature high telomerase activity in many organs, in contrast to humans (Prowse and Greider, 1995). Interestingly, inbred mice have long (Kipling and Cook, 1990) while wild mice have short telomeres, suggesting telomere length does not affect organismal longevity (Hemann and Greider, 2000). In fact, telomerase activity appears to be crucial for the normal functioning of highly proliferative organs in mice (Lee *et al.*, 1998). Therefore, telomere length and/or telomerase activity do not explain why humans age slower than other primates and mice. Yet it helps explain why mice have a much higher cancer incidence than men (Blasco, 2003).

As mentioned in chapter 2.4., the relation between RS and organismal ageing is unproven. Thus, whether telomere shortening plays a role in human ageing is debatable. As with replicative potential, telomere length *in vivo* is very heterogeneous (Serra and von Zglinicki, 2002; Takubo *et al.*, 2002). Telomere shortening *in vivo* has been reported in liver cells (Aikata *et al.*, 2000), lymphocytes (Mariani *et al.*, 2003), skin cells (Lindsey *et al.*, 1991), blood (Iwama *et al.*, 1998), and colon mucosa (Hastie *et al.*, 1990). Other studies found weak correlations between donor age and telomere length (Allsopp *et al.*, 1992; Kammori *et al.*, 2002), while some studies found no correlation (Mondello *et al.*, 1999; Renault *et al.*, 2002; Serra and von Zglinicki, 2002; Takubo *et al.*, 2002). Finally, long telomeres have been found in cells from centenarians (Franceschi *et al.*, 1999). Taken as a whole, these results indicate that telomere length varies widely amongst individuals and between different tissues and that telomere shortening might occur in some tissues *in vivo*.

Most, not all, human somatic tissues have no detectable telomerase activity (reviewed in Collins and Mitchell, 2002). In the bone marrow, hematopoietic cells express telomerase. Telomerase activity is higher in primitive progenitor cells and then downregulated during proliferation and differentiation (Chiu *et al.*, 1996). Other reports associate, normally low, levels of telomerase activity with human stem cells (Sugihara *et al.*, 1999), though not mesenchymal stem cells (Zimmermann *et al.*, 2003). Telomerase activity has been detected in several normal human somatic proliferating cells: for instance, skin (Harle-Bachor and Boukamp, 1996; Taylor *et al.*, 1996; Yasumoto *et al.*, 1996), immune system (Counter *et al.*, 1995; Morrison *et al.*, 1996; Weng *et al.*, 1997), and colorectal tissues (Tahara *et al.*, 1999). Human germ cells have been found to express hTERT (Kilian *et al.*, 1997).

hTERT expression appears to be regulated by the oncogene transcription factor c-Myc (Wu *et al.*, 1999). This is intriguing and may relate telomerase to ageing, particularly since c-Myc is also involved in WRN regulation (Grandori *et al.*, 2003). Yet other players have been suggested as regulators of hTERT: Sp1, Mad, and Max (Wick *et al.*, 1999; Xu *et al.*, 2001). Therefore, further data is necessary to elucidate the regulation of hTERT expression.

Dyskeratosis congenita is an inherited disease involving skin and bone marrow failure (reviewed in Marrone and Mason, 2003). It is caused by a mutation in the DKC1 gene. Intriguingly, the protein encoded by DKC1, dyskerin, is a component of telomerase. Mutations in the RNA component of telomerase are associated with the autosomal dominant form of dyskeratosis congenita (Vulliamy *et al.*, 2001). Families with this form of the disease are more severely affected in later generations, suggesting telomere shortening mechanisms are involved. Features of dyskeratosis congenita include bone marrow failure, which is the most usual cause of death, abnormal skin pigmentation, leukoplakia and nail dystrophy (Knight *et al.*, 1998). The role of stem cells has also been suggested (Mason, 2003).

As judged from the phenotype of dyskeratosis congenita, telomeres are crucial in rapidly proliferating tissues but it is unclear whether telomere shortening is involved in human ageing. It is possible, however, that telomere shortening is involved in age-related deterioration. Despite having active telomerase, the telomeres of lymphocytes shorten with age (Pan *et al.*, 1997). A decline in telomerase activity was also found in blood mononuclear cells with age (Iwama *et al.*, 1998). Though mean telomere length at birth does not correlate with longevity in birds, telomere shortening in erythrocytes inversely correlates with bird longevity. Telomere shortening in a variety of tissues also correlates, though to a lesser extent, with mammalian longevity (Haussmann *et al.*, 2003; Vleck *et al.*, 2003; Figure 17). In fact, a correlation between erythrocyte longevity and organismal longevity was previously shown, suggesting that cells, in this case erythrocyte stem cells, from long-lived animals divide fewer times (Rohme, 1981). The role of telomeres in RS has led to suggestions that telomerase can be used as an anti-ageing therapy (reviewed in Fossel, 1996; Blasco, 2003).

Lastly, it is unquestionable that RS and SIPS are important tools to study cancer and develop anti-cancer treatments (reviewed in Campisi *et al.*, 2001; Blasco and Hahn, 2003; Hahn, 2003; Lee and Schmitt, 2003; Wang *et al.*, 2003).

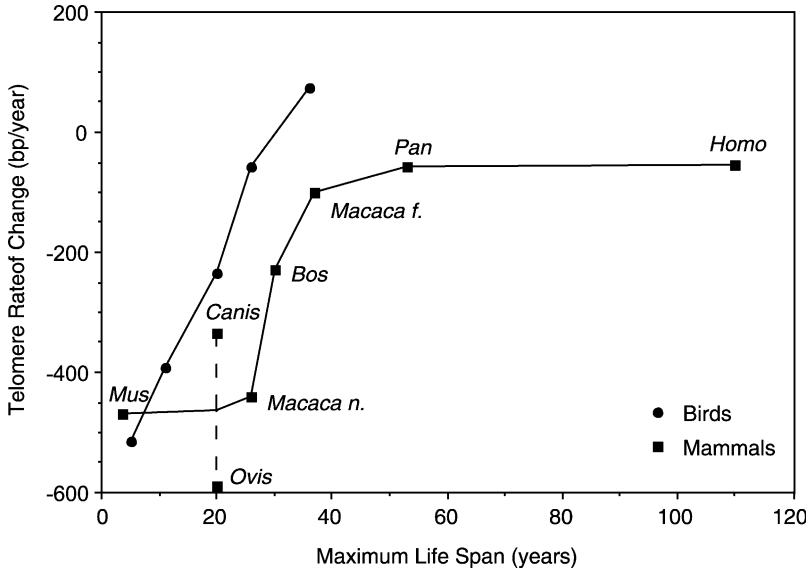

Figure 17: The relationship between rate of change in mean telomere length (base pairs lost [negative numbers] or gained [positive numbers] per year) in various tissues and life span for five birds (circles) and eight mammals (boxes). For each class, lines connect the points (or mean of the points for species with the same lifespan). Taken from Vleck *et al.*, 2003. Also refer to Haussmann *et al.*, 2003 for data sources.

CHAPTER 4: MECHANISMS OF SIPS

4.1. From DNA damage to SIPS

So far, our focus has been on premature cellular senescence induced by damaging agents. Yet SIPS can be induced by several oncogenes such as *ras* (Serrano *et al.*, 1997). $p16^{INK4a}$ or $p21^{WAF1}$ overexpression also induces senescence (McConnell *et al.*, 1998). This further demonstrates the connection between cellular senescence and cancer and suggests that SIPS can also be a tumour-protective mechanism, like RS was proposed to be. As such, the pathways involved in SIPS are of particular interest to research the mechanisms behind cancer and ageing, the two of which may be related (Miller, 1991; Anisimov, 2001).

In parallel to what happens in RS, pRb plays a crucial role in SIPS. In fact, cells expressing E7, that blocks pRb activity, do not undergo SIPS as a consequence of H_2O_2 treatment (Chen *et al.*, 2000b). These results suggest that the phosphorylation status of pRb is critical for G1/S growth arrest and the appearance of SIPS. Also important in SIPS is p53. DNA damage activates p53 which in turn triggers $p21^{WAF1}$ (Di Leonardo *et al.*, 1994; Chen *et al.*, 1998). $p21^{WAF1}$ itself appears an important player since HDFs lacking $p21^{WAF1}$ failed to arrest the cell cycle in response to DNA damage (Brown *et al.*, 1997). Yet blocking p53 through E6 expression does not prevent SIPS (Dulic *et al.*, 2000). p53's protein level increases shortly after H_2O_2 stress but returns to basal level at 48 hrs. On the contrary, $p21^{WAF1}$'s level remains elevated for at least 21 days (Chen *et al.*, 1998). These results suggested that p53 may not be a crucial player in SIPS. Although p53-independent induction of $p21^{WAF1}$ has been reported (Tahara *et al.*, 1995), $p21^{WAF1}$ action through DNA damage is probably regulated by p53 (Michieli *et al.*, 1994). Nevertheless, the possibility exists that $p21^{WAF1}$ induces SIPS without p53. In addition to the p53/$p21^{WAF1}$ pathway, $p16^{INK4a}$ is also involved in SIPS and $p16^{INK4a}$ is induced by DNA damage (Robles and Adami, 1998; Shapiro *et al.*, 1998). Taken together, these results suggest that DNA damage triggers a set of molecular pathways similar to those involved in RS (Figure 18). Interestingly, *ras*-induced senescence also appears to involve the p53 and $p16^{INK4a}$ pathways (Serrano *et al.*, 1997; Ferbeyre *et al.*, 2002), again arguing in favour of seeing cellular senescence as an anti-cancer mechanism. Yet other mechanisms may play a role: the anticancer drug cisplatin can cause SIPS in cancer cells independently of p53, $p21^{WAF1}$, and $p16^{INK4a}$ (Wang *et al.*, 1999).

Although it is undeniable that the p53/$p21^{WAF1}$ and pRb/$p16^{INK4a}$ pathways are involved in SIPS, it is unclear how they are activated in response to DNA damage. The mitogen-activated

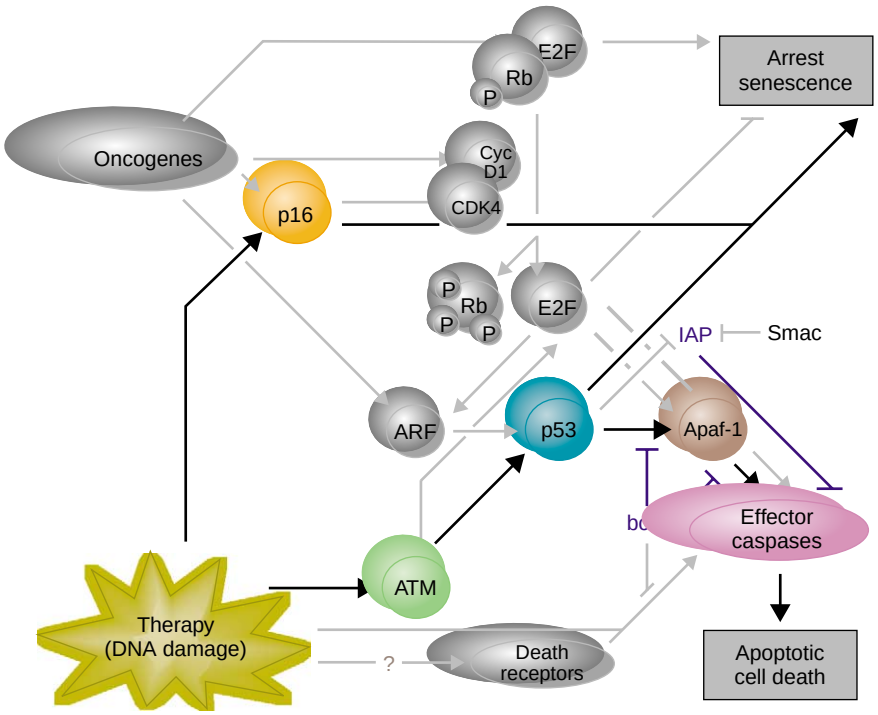

Figure 18: DNA-damage pathways into cell-cycle exit. DNA-damage (black lines) can induce $p16^{INK4a}$ or may be sensed by ATM or related kinases which then activate p53. $p16^{INK4a}$ and p53 cooperate to induce SIPS. Apoptotic pathways may also be activated through p53. Adapted from Lee and Schmitt, 2003.

protein kinase (MAPK) family was shown to be involved in *ras*-induced senescence by activating p53, 21^{WAF1}, and $p16^{INK4a}$ and triggering SA β-gal activity (Lin *et al.*, 1998b). JunB may also be a transcriptional activator of $p16^{INK4a}$ (Passegue and Wagner, 2000). As for p53, many signalling pathways activated by stress, such as ROS, can contribute to p53's activation (reviewed in Martindale and Holbrook, 2002). The tumour suppressor PLM may regulate p53 in response to oncogenic stimuli (Pearson *et al.*, 2000). Proteins involved in DNA damage recognition, such as ATM, may be involved in p53 activation (Banin *et al.*, 1998; Canman *et al.*, 1998; Rouse and Jackson, 2002). In fact, ATM has been hypothesized as a sensor of stress (Rotman and Shiloh, 1997). As happens in RS, the mRNA levels of the oncogene *c-fos* also decrease in SIPS (Dumont *et al.*, 2000a), suggesting a loss in the activity of AP-1, which has been hypothesized to activate p53 in RS (Irving *et al.*, 1992). Other results, however, deem such conclusions as unlikely and indicate that AP-1 and *c-fos* are not critical for cellular senescence (Lucibello *et al.*, 1993). The transcription factor NF-κB has also been suggested to upregulate p53 (Wu and Lozano, 1994; Dumont *et al.*, 1999). NF-κB itself may be activated, in some, but not all, cell lines, in response to UVB (Helenius *et al.*, 1999) or H_2O_2 (reviewed in Li and Karin, 1999; Bowie and O'Neill, 2000). On the other hand, ROS may activate p53 directly by modulating the redox status of a set of cysteins in the DNA-binding domain (Meplan *et al.*, 2000). Despite the knowledge of multiple pathways that respond to stress (reviewed in Martindale and Holbrook, 2002), the exact cascade of events leading to SIPS is unknown.

One important player may be transforming growth factor-β1 (TGF-β1). Briefly, the TGF-β family is involved in multiple functions including development, differentiation, morphology, and cellular proliferation (reviewed in Alevizopoulos and Mermod, 1997; Padgett *et al.*, 1998). Immortalization has been associated with loss of response to TGF-β and incubation of HDFs with TGF-β induces a senescent phenotype (Sorrentino and Bandyopadhyay, 1989; Herrera *et al.*, 1996; Katakura *et al.*, 1999). SIPS triggers the release of TGF-β1 while incubation with antibodies against TGF-β1 or its receptor II represses SIPS (Frippiat *et al.*, 2001). Intriguingly, TGF-β1 induces H_2O_2 production in HDFs, suggesting that a positive feedback loop may be formed (Thannickal and Fanburg, 1995). TGF-β1 can downregulate the CDK2 (Hu *et al.*, 2001). Not surprisingly, pRb interacts with the TGF-β family. Not only TGF-β can alter the phosphorylation status of pRb (Laiho *et al.*, 1990), but pRb can regulate the expression of TGF-β1 (Kim *et al.*, 1991).

One possible link between DNA damage and TGF-β involves a member of the MAPK family: $p38^{MAPK}$, which was shown to be involved in growth arrest in response to oxidative stress (Kurata, 2000). Activation of p53 by $p38^{MAPK}$ has also been reported (Bulavin *et al.*,

1999). Clearly, $p38^{MAPK}$ is activated shortly after stress (Chen *et al.*, 2001a; Kabuyama *et al.*, 2001; Volonte *et al.*, 2001) and can remain active for 72 hrs after stress (Frippiat *et al.*, 2002). One hypothesis is that $p38^{MAPK}$ activates the activating transcription factor-2 or ATF-2 (Cheong *et al.*, 1998; Waas *et al.*, 2001), which in turn can interact with pRb (Kim *et al.*, 1992). The interaction of ATF-2 and pRb has been demonstrated by immunoprecipitation (Frippiat *et al.*, 2002) but exactly how pRb interacts with ATF-2 is unclear. Nonetheless, evidence suggests that ATF-2 mediates pRb's regulation of other genes while at the same time ATF-2 is important in the transcription of pRb (Park *et al.*, 1994). TGF-β1 would then be overtranscribed by the concerted action of ATF-2 and pRb, as has been shown to occur to TGF-β2 (Kim *et al.*, 1992). In turn, TGF-β1 can activate $p38^{MAPK}$ and consequently ATF-2 (Hanafusa *et al.*, 1999). These results led to the suggestion of a regulatory loop between TGF-β1 and $p38^{MAPK}$ (Figure 19). Indeed, antibodies against TGF-β1 prevented the sustained, but not short term, phosphorylation of $p38^{MAPK}$ and ATF-2 under SIPS, while inhibition of $p38^{MAPK}$ or ATF-2 prevents overexpression of TGF-β1 and represses SIPS. It was also shown that the appearance of SA β-gal activity in H_2O_2-induced SIPS depends on the activation of $p38^{MAPK}$ and ATF-2 (Frippiat *et al.*, 2002). Moreover, a recent study found that $p38^{MAPK}$ inhibition repressed SIPS and delayed RS. A differential role of pRb was also suggested. The same study suggested that unidentified factors act upstream of $p38^{MAPK}$ in response to H_2O_2 because activation of $p38^{MAPK}$ continued even after exposure to H_2O_2 (Iwasa *et al.*, 2003), yet it may be that the regulatory loop between TGF-β1 and $p38^{MAPK}$ activated $p38^{MAPK}$. Importantly, though, pRb's regulation of TGF-β1 and even TGF-β1's functions vary between different cell lines (Kim *et al.*, 1991; Massague, 1998).

Although the $p53/p21^{WAF1}$ and $pRb/p16^{INK4a}$ pathways are involved in activating SIPS, it is likely that other pathways also have the ability to cause SIPS. At least in some cell lines, one of those pathways may involve $p38^{MAPK}$ and TGF-β.

4.2. The telomeres

Although telomeres are a key factor in RS, when we began our work, their role in SIPS was a subject of debate. Of importance is the work of Thomas von Zglinicki, who initially showed that HDFs cultured at 40% O_2 undergo SIPS after a maximum of three CPDs. Telomere shortening of cells under hyperoxia was around 500 bp per population doubling (PD) when compared to the 90 bp/PD shortening of controls. A telomere cut-off length of 4 kbp at which cells were growth arrested was also found. These results led to the suggestion that telomere shortening is the signal that initiates senescence (von Zglinicki *et al.*, 1995).

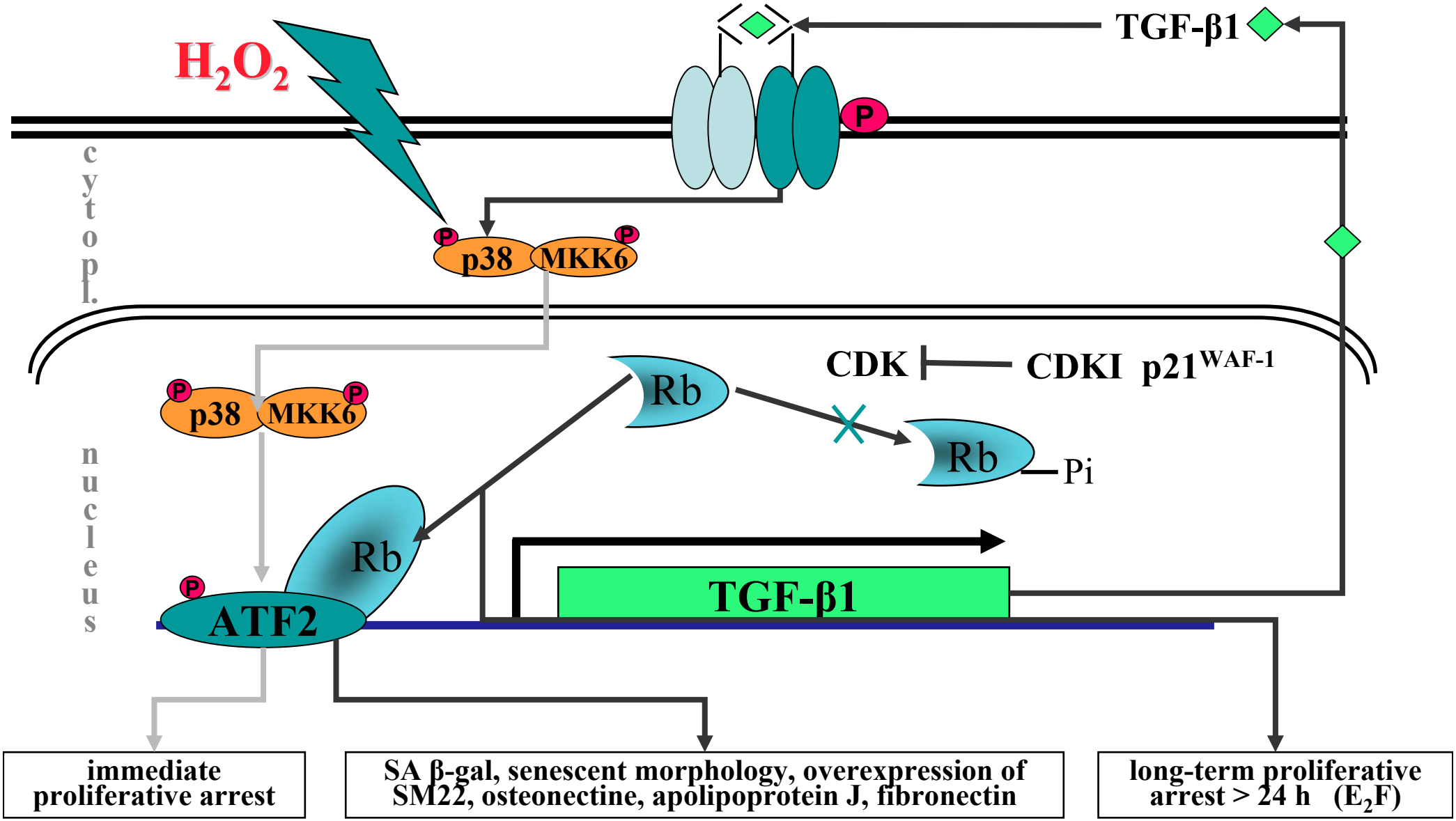

Figure 19: Representation of the closed regulatory loop that induces and maintains the biomarkers of SIPS at a long term after H_2O_2 stress. The early events are indicated with light grey arrows and the late events in dark grey. Adapted from the thesis of Christophe Frippiat.

It was already known at the time that DNA damage could result in growth arrest (Di Leonardo *et al.*, 1994). Yet further results from von Zglinicki and his colleagues suggested that telomeres are important "sensors" of DNA damage. Single and double strand breaks are the hallmark of oxidative damage resulting from ionizing radiation or H_2O_2 (reviewed in Beckam and Ames, 1998). As a result of different types of oxidative damage, single-strand regions--breaks, overhangs, and gaps--appear to accumulate more often in the telomeres than in other regions of the genome (Petersen *et al.*, 1998). These results suggested that telomeres are "guardians of the genome", triggering senescence in both RS and SIPS (von Zglinicki and Saretzki, 1997). It was also hypothesized that different rates of telomere shortening within cultures would determine the heterogeneity of cell populations (Rubelj and Vondracek, 1999). In addition, oxidative stress mediated the production of single-strand damage that would lead to growth arrest through the p53/p21^{WAF1} pathway. Furthermore, the introduction of telomeric oligonucleotides into HDFs induced a growth inhibition also based on the p53/p21^{WAF1} pathway (Saretzki *et al.*, 1999).

In its essence, the hypothesis relating telomeres to SIPS was that oxidative damage would cause single-strand damage to telomeric DNA. Unlike damage to the genome, damage to the telomeres cannot be repaired in normal HDFs since these lack telomerase activity, resulting in telomere shortening and single-strand DNA that would trigger SIPS through the p53 pathway (reviewed in von Zglinicki, 1998).

Shortly after we began our work, one experiment in our laboratory raised doubts on the influence of telomere shortening in SIPS. HDFs exposed to four subcytotoxic t-BHP stresses every two PDs underwent a telomere shortening of 245 bp/PD, in contrast to 107 bp/PD in controls. Accelerated telomere shortening was also witnessed in the PD following five repeated stresses with t-BHP or a single H_2O_2 stress: respectively, 381 and 322 bp. Telomere shortening in subsequent PDs was similar to controls: 108 bp/PD in the t-BHP-treated HDFs, 93 bp/PD in the H_2O_2-treated HDFs, and 109 bp/PD in controls. Since telomeres did not reach their critical length, it was hypothesized that telomere shortening was a consequence rather than a cause of SIPS. Compared to controls, about 20% of cells could proliferate after stress, meaning that these cells had to compensate for the growth-inhibited ones, termed compensatory cycling. Having cells divide more often would result in an increase in telomere shortening, and thus telomere shortening would be a result rather than a cause of SIPS. These results indicated that the role of telomeres in SIPS might be overestimated and suggested that other mechanisms may be at work (Dumont *et al.*, 2001). Earlier results had already shown that senescence could be induced without telomeres reaching their critical lengths (Michishita *et al.*, 1998; Suzuki *et al.*, 2001b).

More recent calculations on the rate of telomere shortening in HDFs under oxidative stress showed that compensatory cycling is not sufficient to explain the observed telomere shortening under mild oxidative stress, and so oxidative stress accelerates telomere shortening (Toussaint *et al.*, 2002a; von Zglinicki, 2002). Yet since senescence could be observed in the absence of critically short telomeres, the importance of telomeres in SIPS was unclear. Addressing the role of telomeres in SIPS was one of the aims of our work and will be thoroughly discussed herein.

CHAPTER 5: COMPUTATIONAL METHODS

5.1. From genes to ageing

An old debate in the field of gerontology is whether genetic or environmental factors are predominant in ageing. Irrespectively of what factors are predominant, in humans, age-related degenerative changes and diseases can result from genetic or environmental factors, or a combination of both. For example, the chances of developing lung cancer can be increased by genetic and environmental factors (Reif, 1981). Therefore, age-related changes derived from both intrinsic and extrinsic factors deserve medical attention and both must be studied for us to have a complete picture of the ageing phenotype. Indeed, there is a great variability in the human ageing phenotype as a consequence of both genetic and environmental factors (reviewed in Finch, 1990). In addition to developing methods to study age-related changes mostly derived from extrinsic factors--i.e. using SIPS--, our aim was also to develop tools and strategies to study the genetic basis of human ageing.

Many others have tried to study the genetics of ageing by way of approaches which will be discussed succinctly. One common strategy is to search life-extending genetic manipulations in model organisms (reviewed in Johnson and Shook, 1997) such as *Drosophila* (reviewed in Tower, 2000), *C. elegans* (reviewed in Johnson, 2002), or mice (for example: Flurkey *et al.*, 2001). These studies have already found a few genes that may play a role in human ageing. The problem, as mentioned in chapter 1.5., is that genetics of ageing in model organisms may or may not be similar to the genetics of ageing in humans. For example, mutations in the human *WRN* appear to accelerate ageing (Goto, 1997 for arguments) while mutating the *WRN* homologue in mice has no visible effect on their ageing process (Lombard *et al.*, 2000; Wang *et al.*, 2000a), despite WRN being involved in murine tumour suppression (Lebel *et al.*, 2001 & 2003). Another approach is to attempt to locate genes in humans that influence ageing by studying, for example, families with exceptional longevity (Puca *et al.*, 2001). Though potentially useful, these studies may be secondary to ageing for the genes located may influence longevity without affecting ageing (Perls *et al.*, 2002). In fact, many genes may influence age-related changes (Martin, 1982). Although studying genes that affect one or a few age-related pathologies without affecting rate of ageing is medically sound, our priority is to understand the genetics of the ageing process rather than individual diseases. Even SIPS, though based on extrinsic factors, can be helpful in understanding the genetics of ageing for genes involved in stress resistance might be involved in the ageing process. Yet, of course, extrapolating from *in vitro* senescence to

organismal ageing is not straightforward, as mentioned in chapter 2.4. Overall, studying human ageing is limited by the available models, as pointed out in chapter 1.5.

Scientific discovery has always been limited by the available technologies. The recent sequencing of the human genome has unleashed a flood of data (Lander *et al.*, 2001; Venter *et al.*, 2001). Due to our lack of knowledge regarding the human ageing process, such large amounts of data can be particularly useful to study the genetics of ageing. So far we only know of a few genes that modulate rate of ageing in animals, and probably only one or two in humans--i.e. the progeroid genes. Yet findings such as *WRN* show the extraordinary influence of genes on the ageing phenotype. Discovering more genetic players in the human ageing process would be a major breakthrough and a step forward in uncovering the genetic basis of human ageing. Therefore, we wanted to develop new approaches to study the genetics of human ageing based on the incoming tidal wave of data. In addition to genomic sequence--i.e. DNA sequence--, the life sciences are also being flooded by protein sequences, macromolecular structures, and gene expression data. To organize, analyse, and interpret biological data, bioinformatics was born (reviewed in Luscombe *et al.*, 2001; Yaspo, 2001).

5.1.1. Comparative genomics

One powerful way to analyze and interpret genomes is by comparing different genomes, known as comparative genomics (reviewed in Clark, 1999; O'Brien *et al.*, 1999; Wei *et al.*, 2002; Ureta-Vidal *et al.*, 2003). The basic assumption when comparing two genomes is that they had a common ancestor and so each base pair in each genome results from a combination of the ancestral genome plus evolution. Comparative genomics can be employed at a holistic level. Briefly, structural genomic features such as genome size, nucleotide statistics (G+C content and G+C distribution across the genome), gene density, and distribution of DNA repeats can be determined. Also relevant is the analysis of synteny, which describes regions of two genomes that share similar genes and DNA sequences and thus are likely to have evolved from a common evolutionary ancestor. Syntenic regions give clues on genome organization and evolution.

Comparisons can also focus on coding regions (reviewed in Wei *et al.*, 2002; Ureta-Vidal *et al.*, 2003). Unlike bacteria, eukaryotes have introns and large non-coding intergenic regions, making gene prediction much more difficult. Since mutations accumulate less often in gene-coding regions, the identification of conserved sequences between different genomes often reveals coding regions. Indeed, new genes have already been discovered based on comparative genomics between the mouse and human genomes (Dehal *et al.*, 2001; Pennacchio *et al.*, 2001).

When several bacterial genomes became available--not only from different species but also from different sub-species and even from different strains--, comparisons between different genomes allowed a better understanding of gene function (reviewed in Yaspo, 2001; Wei *et al.*, 2002). One approach is to build a phylogenetic profile by determining the presence or absence of a protein across many genomes. Functionally related proteins are expected to have similar profiles and thus can be grouped together. Although the method does not solve the problem of false negatives, functionally similar proteins do tend to have similar phylogenetic profiles (Pellegrini *et al.*, 1999). Hints about protein function and protein-protein interactions in an organism can also be studied by finding protein fusions in other organisms (Marcotte *et al.*, 1999a). Another approach involves a detailed comparison of specific pathways. In one example, researchers compared DNA repair pathways from the bacterium *Escherichia coli* and *S. cerevisiae* across different genomes in order to understand the evolution of repair systems (Aravind *et al.*, 1999).

To make comparative genomics possible, a variety of algorithms and computer programs exist, from tools to perform whole-genome alignments to programs to produce alignments between subregions of sequences, called local alignments (reviewed in Wei *et al.*, 2002; Couronne *et al.*, 2003; Frazer *et al.*, 2003; Pennaccio and Rubin, 2003; Ureta-Vidal *et al.*, 2003). Of course that comparative genomics is not without its limitations, particularly when applied to the large vertebrate genomes. One major problem arises from inaccurate biological information added or missing from genome sequence, called genome annotation. For example, common errors arise in gene prediction. Another type of limitation results from the unpredictable nature of biological systems. Two structurally-related genes can have different functions and vice-versa (reviewed in Yaspo, 2001). Despite these pitfalls, comparative genomics is becoming an increasingly important tool for deciphering gene function and understanding biological systems.

5.1.2. Transcriptional regulation

One of the surprising discoveries from the sequencing of mammalian genomes is their extraordinary similarity. For example, DNA sequences between humans and chimpanzees share about 95% of base pairs (Britten, 2002) and probably chimpanzees have our same set of genes (Wade, 2001). Even mice and humans share 97% of their genes, according to the lowest of the latest estimates (Mural *et al.*, 2002; Waterstone *et al.*, 2002). What appears to determine the differences between humans and chimpanzees is not gene content *per se* but rather when and how the genes are expressed (Enard *et al.*, 2002). In other words, the genes may well be similar

between humans and other animals, but they are used or transcribed differently and the way they are transcribed determines the differences between humans and other animals (reviewed in Levine and Tjian, 2003).

In eukaryotes, transcription begins with the assembly of a large complex of proteins, including RNA polymerase II, called transcription initiation complex (TIC). The TIC is assembled in a region called the "promoter" or "core promoter" that is located upstream--i.e. is in the 3'→5' direction--of the first nucleotide to be transcribed; this site where transcription of RNA begins is called transcription start site (TSS) (Hampsey, 1998; Lemon and Tjian, 2000; Figure 20).

Transcriptional regulation is a complex process that only recently began to be understood (reviewed in Arnone and Davidson, 1997; Fickett and Wasserman, 2000). Importantly, the regulatory program in eukaryotes is defined in the non-coding genome sequence, largely in the form of *cis*-regulatory sequences that are critical in development and cellular differentiation. These elements are sequence-specific targets of DNA-binding proteins called transcription factors (TFs) that, together with proteins that bind them, control gene activity. Both positive and negative *cis*-regulatory interactions exist, called, respectively, enhancers and silencers. Negative regulators can shut down the TIC but normally they work by disrupting positive regulators. These regulatory elements are normally located within 1,000 bp upstream of the TSS, though some elements can be thousands of bp away. Regulatory elements can also be present within introns downstream of the TSS (Figure 21). Although components of the TIC can modulate gene expression (Holstege *et al.*, 1998), TFs are the major players in transcriptional regulation (Coulson and Ouzounis, 2003 for arguments).

In brief, TFs work together to control gene expression (reviewed in Arnone and Davidson, 1997; Fickett and Wasserman, 2000). The binding specificity of TFs, not their arrangement, is critical to control gene expression. A cluster of TF binding sites (TFBS) where any TF affects gene expression is called a module. From a structural point of view, TFs probably cooperate through DNA looping. In fact, certain proteins, such as Sp1, appear to help modules interact by promoting or stabilizing loops (Pascal and Tjan, 1991). Combining regulatory elements from the gut-specific *Endo 16* gene and from the skeletogenic *SM50* gene, scientists obtained a combined gut and skeletogenic mesenchyme pattern of expression, showing how two modules can cooperate (Kirchhamer *et al.*, 1996). Yet a gene's regulation can occur through single or multiple modules. Lastly, chromatin modifications can prevent transcription by blocking access to the genome and, though the mechanisms involved are unclear, have profound effects on gene expression (Felsenfeld and Groudine, 2003). Although TFs can be seen as

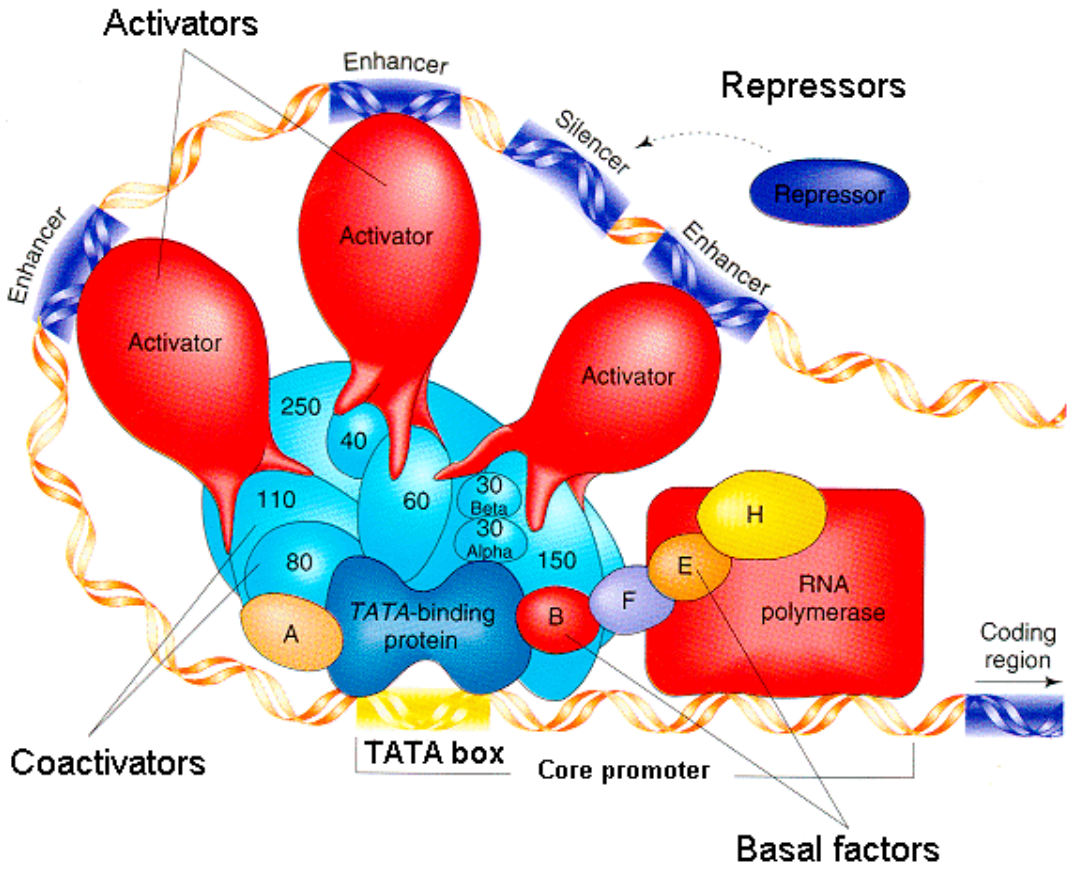

Figure 20: The eukaryotic promoter consists of core elements, which include the TATA box and other DNA sequences that define transcription start sites, and regulatory elements, which either enhance or repress transcription in a gene-specific manner. Regulatory elements bind gene-specific factors, which affect the rate of transcription by interacting, either directly or indirectly, with components of the transcriptional machinery. Adapted from Tjian, 1995 and Hampsey, 1998.

upstream of other genes from a genomic perspective, it is also important to have in account protein-protein interactions as well as the regulatory interactions between TFs that give rise to complex regulatory networks. One example of a well-characterized regulatory region is that of the *Endo 16* gene from the sea urchin (Yuh *et al.*, 1998; Figure 22).

As a whole, TF target sites in the regulatory regions of a gene determine the inputs to which the gene will respond and the target sites of a TF determine the response that TF will generate. Since transcriptional regulation is located in the genetic information, bioinformatics is also attempting to tackle regulatory networks. Although regulatory analysis requires molecular manipulations such as analysis of DNA-protein interactions *in vitro*, mutations, and synthetic expression constructs, computational tools are becoming increasingly important in understanding regulatory networks. Algorithms can locate putative TFBS in genome sequence, though these continue to suffer from an excessive number of false positives (reviewed in Stormo, 2000; Ohler and Niemann, 2001; Pennacchio and Rubin, 2001). This problem probably results from competitions amongst TFBS as well as changes in chromatin structure that influence the functionality of TFBS (Audic and Claverie, 1998; Fickett and Wasserman, 2000).

Comparative genomics of transcriptional regulation, or phylogenetic footprinting (Tagle *et al.*, 1988), is a branch of comparative genomics also based on the principle that functionally important regions have lower mutation rates (reviewed in Hardison *et al.*, 1997; Ansari-Lari *et al.*, 1998; Hardison *et al.*, 2000; Pennacchio and Rubin, 2001). Consequently, locating conserved non-coding regions may help determine regulatory regions since these appear to have a higher density of regulatory signals (Hardison *et al.*, 1997; Levy *et al.*, 2001). Phylogenetic footprinting has already been successfully used in the discovery of mammalian regulatory elements of genes such as Bruton's tyrosine kinase and β-globin (Hardison *et al.*, 1997), interleukins 4, 5, and 13 (Loots *et al.*, 2000), stem cell leukaemia gene (Gottgens *et al.*, 2000 & 2001), as well as many others (for example: Ellsworth *et al.*, 2000; Leung *et al.*, 2000; Wu *et al.*, 2001). In addition, with the availability of more genomes, phylogenetic footprinting should become gradually more powerful (Hardison *et al.*, 1997; Dubchak *et al.*, 2000; Mouchel *et al.*, 2001). One major problem with phylogenetic footprinting, as well as overall TFBS identification by computational methods, is the presence of false positives. Nevertheless, phylogenetic footprinting followed by experimental identification is an effective method for the identification of transcriptional regulatory regions.

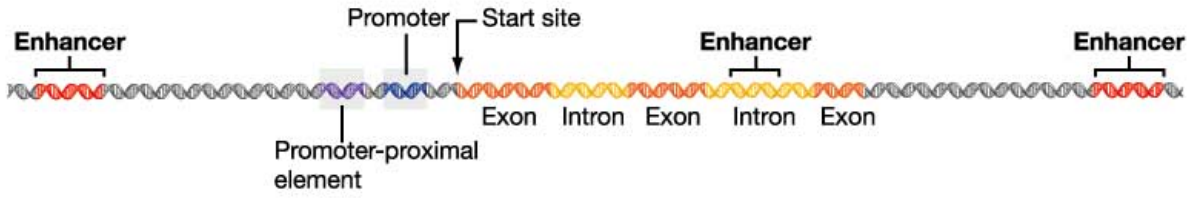

Figure 21: Representative example of a eukaryotic promoter. In biology the term "promoter" has two meanings: it can refer to the region immediately upstream of the TSS (marked "promoter" in the figure) or it can refer to *cis*-acting genetic elements controlling the rate of the TSS (marked "enhancer" in the figure). In this work we use the later definition, unless otherwise noticed, as in the case of this figure. Adapted from Tjian, 1995.

5.1.3. Gene expression studies

One powerful new tool to understand how transcription works is the DNA microarray (reviewed in Lander, 1999 and the subsequent *Nature Genetics* supplement). The most common goal of employing DNA microarrays is to monitor RNA expression levels. One important limitation is the frequent lack of correlation between messenger RNA (mRNA) and protein levels (Gygi *et al.*, 1999), meaning that mRNA levels give information about regulation but not necessarily functionality.

Briefly, DNA microarrays derive from the principle that labelled nucleic acid molecules can be used to interrogate genetic material to determine DNA sequence attached to a solid support (Southern, 1975). The first protocols resembling modern DNA microarrays used DNA robotically spotted to a microscopic glass slide. DNA complementary to the mRNA of each gene to be studied was used. mRNA double-labelled samples were hybridized with the slides and gene expression would then be quantified by measuring the fluorescence on each spot (Schena *et al.*, 1995). Other ways of building DNA microarrays have also been developed, such as DNA microarrays that use samples with complementary DNA or RNA. With recent progresses, DNA microarrays can now feature thousands of genes (reviewed in Bowtell, 1999; Lockhart and Winzeler, 2000).

DNA microarrays can be divided into low and high density microarrays, being the threshold normally set at 400 spots/cm^2 due to a patent of the Affymetrix company. The great advantage of high density microarrays with thousands of genes--often including genes with no known function--is that it is not necessary to know what genes are important in the process under study (Figure 23). Their major limitation is that it can be difficult to analyse the data, results are often less reproducible, and high density microarrays are much more expensive. In contrast, low density microarrays are cheaper, in general yield more reproducible results, and these are easier to analyze since we know the genes under study. Their major limitation is that they require *a priori* knowledge about the process under study and the genes involved (reviewed in Lockhart and Winzeler, 2000; Zammatteo *et al.*, 2002).

If the aim of a study using DNA microarrays is to find, for instance, tumour markers, then examining the genes whose expression change more markedly is an efficient technique. Yet if the goal is to understand a process based on large amounts of gene expression data, then a more careful analysis is needed. Clustering genes whose expression patterns are similar is a typical step in the analysis of a complex process; a large numbers of clustering algorithms exist (reviewed in D'Haeseleer *et al.*, 2000). Genes with similar functions tend to cluster together,

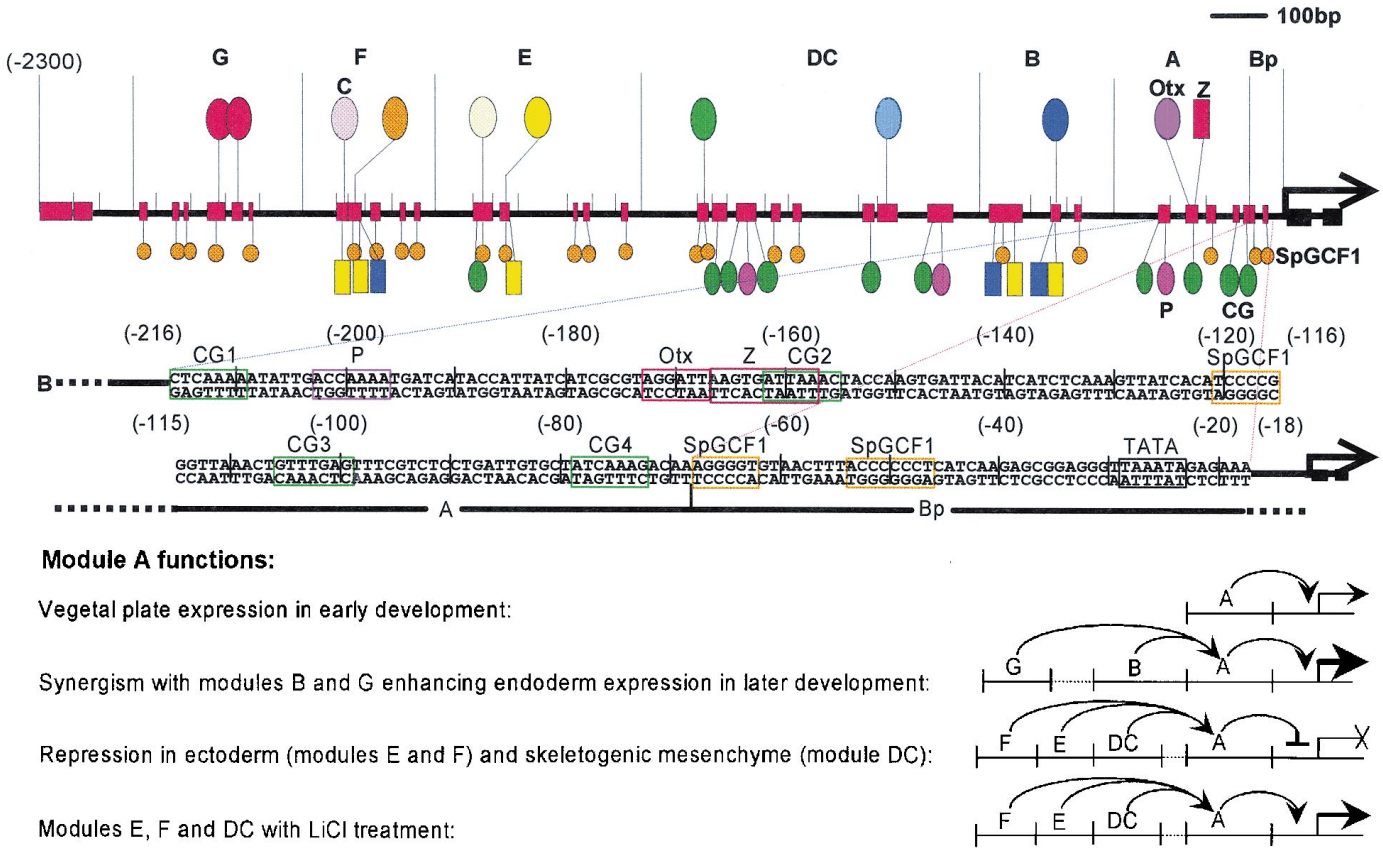

Figure 22: Endo16 *cis*-regulatory system and interactive roles of module A. (A) Diversity of protein binding sites and organization into modular subregions. Specific DNA binding sites are indicated as red blocks; modular subregions are denoted by letters G to A (Bp, basal promoter). Proteins binding at the target sites considered in this work are indicated: Otx, SpOtx-1; SpGCF1; the proteins CG, Z, and P, which are not yet cloned; and protein C (a CREB family protein) in subregion F. Proteins for which sites occur in multiple regions of the DNA sequence (indicated by the black line) are shown beneath. (B) Sequence of module A and location of protein binding sites. Sites are indicated in the same colours as in (A). A fragment containing CG3 and CG4 sites as well as Bp has no endodermspecific activity and services other upstream *cis*-regulatory systems promiscuously; similarly, the Endo16 *cis*-regulatory system functions specifically with heterologous promoters substituted for Bp. Boxed sequences indicate conserved core elements of the target sites, not the complete target site sequences. (C) Integrative and interactive functions of module A. Module A communicates the output of all upstream modules to the basal transcription apparatus. It also initiates endoderm expression, increases the output of modules B and G, and is required for functions of the upstream modules F, E, and DC. These functions are repression of expression in nonendodermal domains and enhancement of expression in response to LiCl. Adapted from Yuh *et al.*, 1998.

making gene expression patterns good indicators of the status of cellular processes. In addition, insights can be gained on genes with unknown functions if they cluster together with known genes (DeRisi *et al.*, 1997; Chu *et al.*, 1998; Eisen *et al.*, 1998; Spellman *et al.*, 1998).

Large amounts of gene expression data obtained under different conditions can be used to identify *cis*-regulatory regions (reviewed in Ohler and Niemann, 2001; Pennacchio and Rubin, 2001). Genes expressed similarly are presumably regulated similarly and therefore may share *cis*-regulatory elements (Chu *et al.*, 1998; Tavazoie and Church, 1998). As such, it is possible to find putative *cis*-regulatory elements in yeast by analysing, with motif discovery programs, the regions upstream of genes clustered together (Tavazoie *et al.*, 1999). Identifying novel motif combinations that affect expression patterns at various stages of yeast development is also possible, leading to a better understanding of the regulatory machinery (Pilpel *et al.*, 2001).

Other technical variants and applications of DNA microarrays exist (reviewed in Lockhart and Winzeler, 2000). One powerful technique for the understanding of transcriptional regulation in yeast is genome-wide location analysis (reviewed in Wyrick and Young, 2002). In brief, the technique involves a DNA microarray with the complete set of yeast intergenic regions (Figure 24). DNA enriched with an antibody against the TF of interest is labelled and hybridized against the DNA microarray revealing which promoters, and often which genes, are regulated by the TF. This approach has been used to gather information about yeast regulatory networks (Simon *et al.*, 2001) as well as to identify new functions of yeast transcriptional activators (Ren *et al.*, 2000; Iyer *et al.*, 2001). Employing genome-wide location analysis to human cells has also been developed using a selection of human promoter sequences. Several genes were identified as potentially activated by E2F including several genes with no known connection to E2F (Ren *et al.*, 2002).

5.2. Systems biology

Understanding a complex process such as human ageing will likely depend on the employment of both computational tools and experimental approaches. The integration of these two forms of biological information requires a systems biology approach (reviewed in Ideker *et al.*, 2001a; Kitano, 2002a & 2002b). Systems biology is based on information obtained from a given biological system under different genetic or environmental conditions. Information is then mathematically treated to construct a model that explains the system. For example, insights into the regulation of galactose use in yeast have been obtained through systems biology by gathering information from gene expression and protein levels, as well as protein-protein and protein-DNA

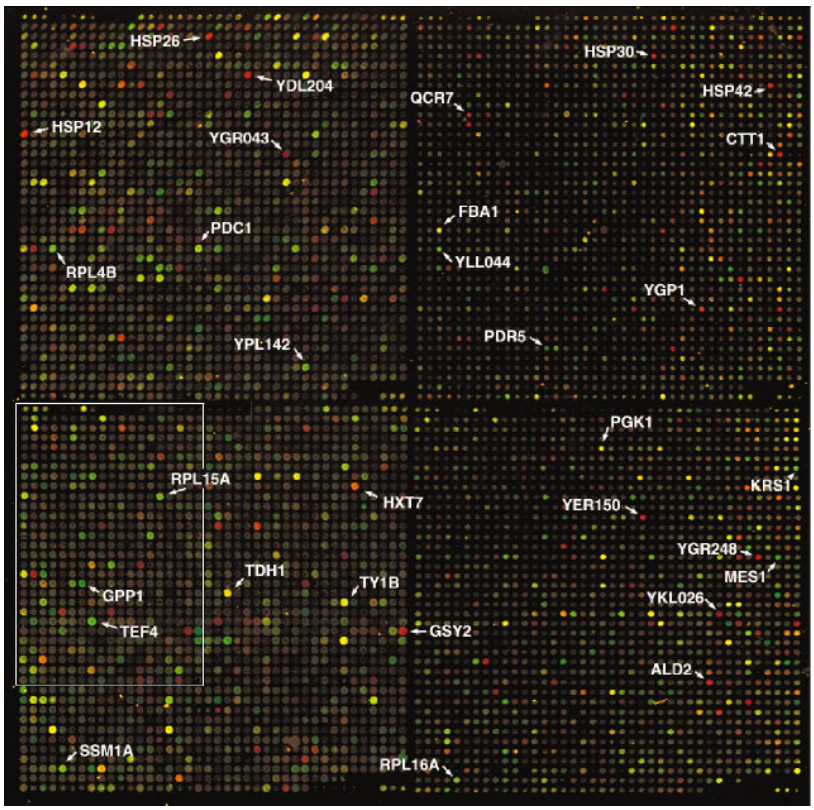

Figure 23: Yeast genome microarray. The actual size of the microarray is 18 mm by 18 mm. A fluorescently labelled cDNA probe was prepared from mRNA isolated from cells harvested shortly after inoculation (culture density of <5 X 10^6 cells/ml and media glucose level of 19 g/liter) by reverse transcription in the presence of Cy3-dUTP. Similarly, a second probe was prepared from mRNA isolated from cells taken from the same culture 9.5 hours later (culture density of ~2 X 10^8 cells/ml, with a glucose level of <0.2 g/liter) by reverse transcription in the presence of Cy5-dUTP. In this image, hybridization of the Cy3-dUTP–labelled cDNA (that is, mRNA expression at the initial timepoint) is represented as a green signal, and hybridization of Cy5-dUTP–labeled cDNA (that is, mRNA expression at 9.5 hours) is represented as a red signal. Thus, genes induced or repressed after the diauxic shift appear in this image as red and green spots, respectively. Genes expressed at roughly equal levels before and after the diauxic shift appear in this image as yellow spots. This image was obtained using a fluorescent scanning confocal microscope. Taken from DeRisi *et al.*, 1997.

interactions under different genetic perturbations (Ideker *et al.*, 2001b). Our understanding of sea urchin development has also been improved by a systems biology approach (Davidson *et al.*, 2002).

One drawback of gene expression data is that although they can be used to identify genes involved in a process, they does not identify the causal relationship between the genes (Kitano, 2002a). Several algorithms exist that attempt to infer a genetic network from gene expression data (reviewed in D'Haeseleer *et al.*, 2000). These methods also follow a systems biology approach since they are based on obtaining a large amount of data under different conditions (Akutsu *et al.*, 1999; Wagner, 2001). Integration of data will be crucial for understanding complex biological systems. For example, understanding transcriptional regulation will likely require the integration of information from phylogenetic footprinting, computer algorithms, and regulatory regions identified from gene expression data (Pennacchio and Rubin, 2001).

Rising from the ongoing genomic revolution, the goals of systems biology are to build a detailed simulation of human cells followed by an identification of all genetic variations, drug responses, and environmental stimuli responses (Kitano, 2002a).

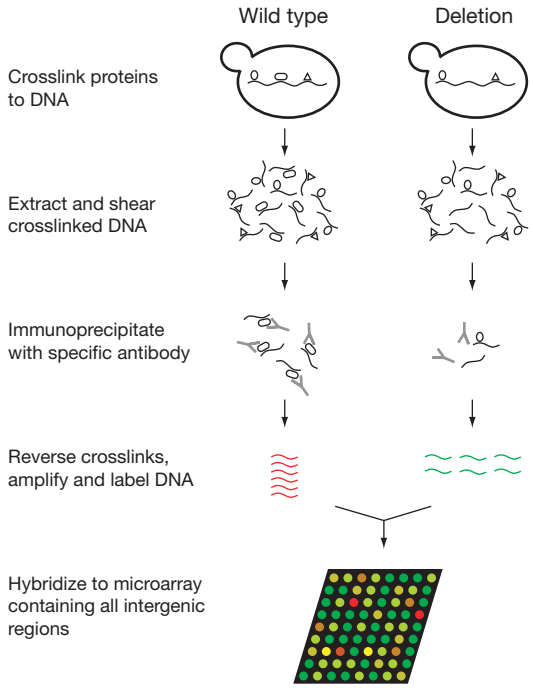

Figure 24: Strategy for analysing genome-wide protein ± DNA interactions. The reference probe can either consist of DNA generated in parallel from a strain bearing a deletion of the gene encoding the protein of interest (as depicted), or of unfractionated genomic DNA amplified and labelled in the same manner. Alternatively, an epitope-tagged version of the protein of interest can be immunoprecipitated with an antibody directed against the epitope. The DNA microarray includes all of the intergenic regions or promoters from the genome. The Cy5/Cy3 fluorescence ratio for each locus reflects its enrichment by immunoprecipitation and therefore, in general, its relative occupancy by the cognate protein. Taken from Iyer *et al.*, 2001.

Aim of the Work

Human ageing derives from a combination of genetic and environmental factors. In this work, we wanted to tackle both. As mentioned earlier, studying human ageing is dependent on the available models, such as animal and cellular models. Integrating studies from several sources has always been problematic, particularly since it is difficult to determine how representative of human ageing each model is. Therefore, one of the goals of this work was to assess models of ageing and determine which are the most appropriate to study the human ageing process.

The different ageing phenotypes found in nature, such as animals that appear not to age, suggest that the evolution of ageing is heterogeneous (reviewed in Finch, 1990). So the first part of our work was to determine how specific the evolution of human ageing is. By analysing the evolution of ageing in humans and evolutionary close taxa, we wanted to have a better view on what organisms are more appropriate to study ageing.

The main reason for choosing cellular biology as a model to study ageing was that it allowed us to focus on human biology rather than that of model organisms. We chose to study senescence in human diploid fibroblasts since the molecular and regulatory pathways involved in RS may be different, for instance, in human, mouse, and chicken cells (Kim *et al.*, 2002). For reasons already mentioned, our main focus was SIPS. Stress resistance *in vitro* correlates with mammalian longevity (Kapahi *et al.*, 1999), so the study of SIPS should not only help clarify extrinsic factors in age-related degeneration, but may also help elucidate the mechanisms of ageing.

Since reactive oxygen species have been proposed to be an important player in ageing (reviewed in Beckman and Ames, 1998), we employed a single 2-hour stress with H_2O_2 to induce SIPS, a model previously established (Chen and Ames, 1994; Chen *et al.*, 1998; Chen *et*

al., 2000b). When we began our work, our laboratory was developing a new SIPS model based on UVB (Chainiaux *et al.*, 2002). Although our priority was oxidative stress with H_2O_2, in collaboration with Florence Chainiaux, we also employed five repeated daily exposures to UVB to induce SIPS.

Telomeres play a critical role in RS (Bodnar *et al.*, 1998 for arguments). Since SIPS also accelerates telomere shortening, telomeres have been proposed as the key signalling process in SIPS (von Zglinicki *et al.*, 1995; von Zglinicki and Saretzki, 1997; von Zglinicki, 1998). At about the time we began our work, other results cast doubts on the importance of telomere shortening in SIPS. Rather than telomeres being a cause of SIPS, it was proposed that telomere shortening was partly a result of compensatory cycling and a consequence of SIPS (Dumont *et al.*, 2001; Toussaint *et al.*, 2002a). Our aim in this work was to elucidate the importance of the telomeres in SIPS.

Since telomerase prevents RS in human cells and appears to stabilizes the telomeres (Blackburn, 2000), by employing a telomerase-positive cell line, we wanted to investigate the effects of telomerase in SIPS. Using hTERT-BJ1 human diploid fibroblasts and BJ controls, we wanted to know whether different mechanisms may be active in provoking SIPS in these two cell lines. Our aim was to study the telomeres to understand the interactions between cellular senescence and stress.

Werner's syndrome appears to cause accelerated ageing (Goto, 1997). If hTERT-BJ1 cells can be seen as having enhanced DNA repair, Werner's syndrome fibroblasts have defective DNA repair systems (Fukuchi *et al.*, 1989). Consequently, we also wanted to establish a model of SIPS to study Werner's syndrome fibroblasts.

One technology deriving from the recent genomic revolution is the DNA microarray (reviewed in Lockhart and Winzeler, 2000). In collaboration with Eppendorf Array Technologies (Namur, Belgium), we employed DNA microarray technology in our research on SIPS.

Computational methods are becoming an integrative part of biological research. We wanted to develop computer methodologies to understand the genetic basis of human ageing. How can researchers take advantage of the recent tools in bioinformatics to study ageing? We wanted to build a strategy for the use of high-throughput computational approaches to the study of ageing that may serve as basis in modelling the human ageing process and developing anti-ageing interventions. This strategy will be developed in a special chapter (11) of this work's discussion.

Results

CHAPTER 6: HUMAN AGEING: EVOLUTION AND RESEARCH MODELS

Since the study of human ageing is based on extrapolations from models, it is critical that we have an assessment of how adequate each model is. The employment of model organisms to study ageing has been, for a long time, a common practice in gerontology. Nonetheless, it is unknown whether mechanisms found in model organisms are representative of the human ageing process. The first part of our work was to address this problem through a comparative biology approach. By comparing the human ageing process with other ageing phenotypes we attempted to have a clearer view on the evolution of ageing and the conservation of the human ageing process.

6.1. Article 1: The evolution of mammalian aging (*Experimental Gerontology*, volume 37, pages 769-775, 2002)

At the time we began our work, the evolution of ageing was thought to be derived from two basic mechanisms: antagonistic pleiotropy, and mutation accumulation (reviewed in Rose, 1991). It was known that changes in extrinsic factors altered a species' mortality and could shape the pace of ageing (Austad, 1988; Austad, 1997a). Yet although the evolutionary theory of ageing offered an explanation for how ageing may have evolved, it did not present specific models to explain all the different ageing phenotypes. In addition, many of the theory's postulates were under attack, such as the inevitability of ageing (reviewed in Finch, 1990). Therefore, we wanted to build a model for the evolution of ageing in humans that explained the phenotype of human ageing when compared to other phenotypes.

In this first article, we compared the ageing phenotypes of different species in order to find trends in the evolution of ageing. Since our goal is to understand the evolution of ageing in humans, we focused on the human taxonomic class, mammals, and closely related taxa.

It was already known that different mammals show a similar ageing phenotype (Finch, 1990). Yet our review of the literature revealed a striking shift in the ageing phenotype of mammals when compared to other classes such as reptiles and amphibians. Although only a fraction of animals had had their ageing phenotype studied, it appeared that the ageing phenotype was somehow more potent in mammals when compared to our reptilian ancestral class. This key insight led us to analyse the evolution of mammals and propose a unique model for the evolution of mammalian ageing. Our model is based on an acceleration of ageing in the primordial mammals whose effects prevail until today.

The implications of our model are multiple and thought-provoking. If indeed mammalian ageing is a more severe phenotype of the process witnessed in reptiles, then studies in reptiles may be useful to understand how genetic and molecular mechanisms can shape ageing and perhaps even reveal potential anti-ageing therapeutic targets. For example, it has been known for a long time that cells taken from the long-lived Galapagos tortoise divide about 110 times (Goldstein, 1974), which is about two-fold the cumulative population doublings human cells can endure. The mechanisms involved are unknown.

If our model is correct, then the evolution of ageing in mammals is a unique phenomenon, meaning mammalian ageing is probably a unique process. Consequently, the organisms best suited to model human ageing are mammals or, more precisely, eutherians. Of course that lower life forms can still be used to gather hints about ageing, but our model clearly establishes that extrapolations into human ageing are inadequate if mammalian models are not employed.

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The evolution of mammalian aging

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Abstract

The incidence of aging is different between mammals and their closer ancestors (e.g. reptiles and amphibians). While all studied mammals express a well-defined aging phenotype, many amphibians and reptiles fail to show signs of aging. In addition, mammalian species show great similarities in their aging phenotype, suggesting that a common origin might be at work. The proposed hypothesis is that mammalian aging evolved together with the ancestry of modern mammals. In turn, this suggests that the fundamental cause of human aging is common to most, if not all, mammals and might be a unique phenomenon. Experimental procedures capable of testing these theories and how to map the causes of mammalian and thus, human aging, are predicted.

Keywords: Aging; Senescence; Evolution; Mammals; Reptiles; Birds; Longevity

1. Introduction

Aging affects all studied mammalian species: from mice living no more than a mere half-a-dozen years to humans capable of living over 120 years (Comfort, 1979, pp. 60–63; Hayflick, 1994, p. 21; Finch, 1994, pp. 150–153). Independent of average and maximum life span and trophic level, all studied mammals show an explicit aging phenotype shortly after their reproductive peek (Finch, 1994, pp. 150–202). In contrast, the data available for the ancestors of mammals (e.g. amphibians and reptiles—who evolved from amphibians 340 million years ago, see Fig. 1) shows both a much more feeble aging phenotype and the presence of apparently non-aging animals (Finch, 1994, 219–221): examples range from *Xenopus* with little over 15 years of maximum life span to Marion's tortoise capable of living over a century in captivity. Studies conducted in frogs failed to indicate any increase in mortality both in the wild (Plytycz et al., 1995) and in captivity (Brocas and Verzàr, 1961). In the case of the yellow-bellied toad, the fact that young individuals (2–4 years-old) were usually found in breeding pods did not prevent the oldest captured animals (of at least 11 years of age) from being fertile and showing little or no signs of aging—no increased mortality was found (Plytycz and Bigaj, 1993). The maximum reported longevity for individuals of this species in captivity is 27 years (Juszczyk, 1987). Studies amongst reptiles, both in the wild and in captivity, also failed to show a significant aging phenotype (Finch, 1994, pp. 219–221). One example is the long-lived Blanding's turtle: these relatively small animals, even though they reach a fixed size, can exceed 75 years of age (Breckle and Moriarty, 1989). In one field study, older turtles were found to have increased clutch size, reproductive frequency, and survivorship (Congdon et al., 2001).

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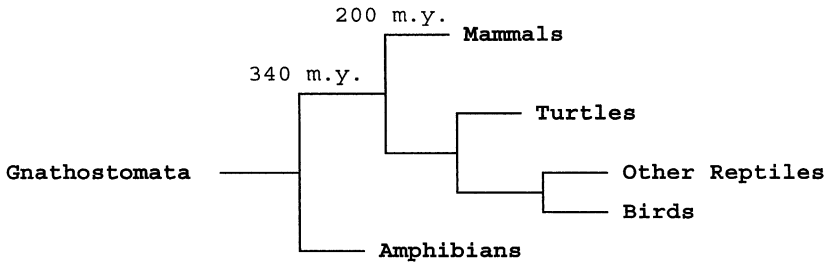

Fig. 1. Simplified version of the evolution of mammals and closely related taxa. Adapted from Maddison and Maddison, 1998.

Similar results have been reported in the three-toed box turtle (Miller, 2001). Even if some of these species eventually age, it is undeniable that aging's incidence and intensity decrease in reptiles when compared to mammals.

Mammalian species show little diversity in their aging phenotype (Finch, 1994, pp. 150–202). All true mammals or eutherians are iteroparous, show a gradual increase in mortality with age shortly after puberty, suffer the effects of aging in a multitude of organs, and, unlike many animals from different phyla, all mammals feature reproductive senescence. Except rodents and marsupials, the increase of mortality with age is within a very short range. This is in contrast with other phyla, such as teleosts, with some animals being semelparous, others featuring gradual aging, and others apparently not aging at all (Finch, 1994, pp. 136–143, 216 and 217). Mammals, in general, have a limited set of teeth, leading to a form of mechanical aging, and have limited cartilage regeneration, which leads to arthritis (Fox, 1938). Other forms of age-related diseases common to mammals include osteoporosis, an exponential increase of cancer with age, and neurodegenerative diseases amongst many of the studied species. Another strange feature of eutherians is the synchronization of many postnatal changes in approximate proportion to the life span, independent of how long this is. The aging pathologies of old mice are roughly the same in old humans or in any well-studied old mammal (Finch, 1994, pp. 150–202, 619). In fact, the aging changes of most well-studied mammalian species appear to be slightly distorted copies of each other, only timed at different paces. Finally, the observation of aged mammals in the wild (Nesse, 1988), such as senescent elephants or post-menopausal whales (Kasuya and Marsh, 1984), suggests that aging did not evolve in these long-lived mammals but instead persisted since their ancestry.

As for reptiles, the available studies not only describe some apparently non-aging species but also show contrasting features with mammals: oocyte regeneration, continuous tooth development, limb regeneration, and increased immune responses to infection. So why the large differences in the intensity of the aging phenotype between mammals and their ancestry? It is unlikely that so many deleterious genes are expressed in mammals and so few in reptiles. What makes mammals more susceptible to aging?

2. The evolution of mammalian aging

Since we witness cases of apparently non-aging species since our aquatic ascendancy (e.g. gnathostomes fishes (Cailliet et al., 2001)) and a sudden widespread burst of aging in mammals, it is implausible that aging developed independently in each species or sub-class of mammals. The simplest explanation to the overall differences between the incidence of the aging phenotype in mammals and their ancestry is that aging evolved to a large degree in the primordial mammals and then persisted throughout the generations until present time. This is the main hypothesis defended in this article.

Current evolutionary theories indicate that the first mammals to evolve from reptiles were small—little more than an inch-long—rodent-like animals, around 200 million years ago (Maddison and Maddison, 1998). Fossil records of early mammals support this view (Crompton and Jenkins, 1968; Luo et al., 2001). It is logical to assume that these first mammals had shorter life spans than their reptilian predecessors as adult body-size, to a certain degree, positively

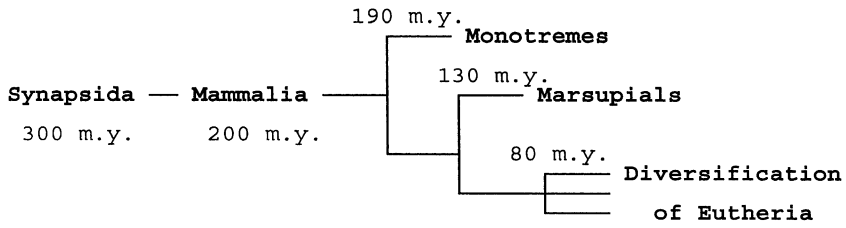

Fig. 2. Synapsids were the first species more closely related to mammals than to reptiles. The first mammals to resemble present species, however, only appeared about 200 million years ago. The branching of mammals began with monotremes (egg-laying mammals) and marsupials. Later, about 80 million years ago, began the branching of eutheria, or true mammals, whose radiation exploded after the mass extinction phenomenon, 65 million years ago. Adapted from Maddison and Maddison, 1998; Finch, 1994, pp. 603–619.

correlates with longevity and rate of aging amongst higher animals (Promislow, 1993; Ricklefs, 1998). Small animals they were until, about 65 million years ago, dinosaurs disappeared and paved the way for mammals (Bryant, 2000, chapter 2). Assuming, as it appears the most likely scenario, that the reptiles from which mammals evolved were slowly or non-aging creatures, we propose mammalian aging flourished during these times. Since aging is unlikely to evolve as an adaptive trait, the process by which this occurred could be: (1) a form of antagonistic pleiotropy as proposed by Williams (Williams, 1957), meaning that mammals possess certain essential function(s) not present in reptiles that as a by-product cause aging; or (2) genetic drift or mutation accumulation leading to the loss or mutation of protective and maintenance genes, caused by the abrupt decrease in life span of these early mammalian species. Female mammals have a limited stock of oocytes, apparently unlike many female reptiles and amphibians, which can even increase their reproductive output with age due to oocyte regeneration. Should this be universally true, it is a case where a loss of function occurred when mammals evolved from reptiles—a loss of function that immediately sets evolutionary pressure for a restriction on life span. Another example is continuous tooth replacement, common in many ancient and modern reptiles but generally absent from mammals (Finch, 1994, pp. 609 and 610). These examples support the hypothesis of short-lived primitive mammals selecting short generation cycles and consequently losing genes whose benefits increase with age as predicted in classical evolutionary theories (Medawar, 1952; Rose, 1991). In addition, the large evolutionary time as small, short-lived animals fostered aging to develop an intensity in mammals not seen in reptiles with similar life spans or body sizes.

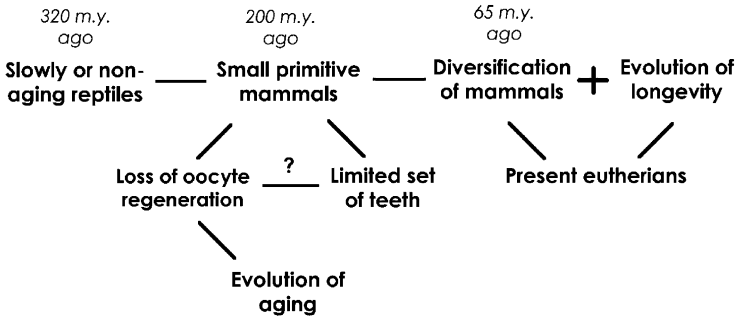

Fig. 3. Overview of the hypothesis for the evolution of mammalian aging. Millions of years as a short-lived species made primordial mammals develop reproductive senescence and consequently what we now call aging. (Although the possibility of a causal relation, one way or the other, between tooth erosion and reproductive senescence should not be ignored.) After the mass extinction in the late Cretaceous (about 65 million years ago), mammals took over the planet: diversifying, evolving new functions, conquering new environments, and generally evolving longevity.

3. Discussion

3.1. A common mammalian cause of aging?

The only documented case of an aging phenotype clearly different from other mammals is the Australian mice *Anthecinus* (Gosden, 1996, pp. 13–30; Finch, 1994, pp. 95–98). So it can be argued that most mammals share their causes of aging: The conquest of the world by mammals began about 65 million years ago, roughly when the dinosaurs disappeared. With exception of egg-laying mammals and marsupials, who diverged from eutherians, respectively, 190 and 135 million years ago (Fig. 2), eutherians evolved from that point to give the species we now know; from fossil records, most species increased in size and therefore evolved longevity, not aging (see Fig. 3). In contrast to the average mammal, elephants have up to six sets of molars (Finch, 1994, p. 199), a clear example of the evolution of a trait that permits a greater longevity. In fact, the first elephants to evolve, about 50 million years ago, were smaller than modern species, indicating elephants evolved longevity and suggesting the existence of senescent elephants in the wild as a consequence of evolving from a faster aging ancestry (see (Haynes, 1993)) for more on the evolution of elephants).

Assuming that aging evolved in the very small and vulnerable first mammals due to their high mortality, it is difficult to imagine greater increases in mortality and thus the aging mechanisms that evolved in mammals more than 65 million years ago remain the same. On the other hand, it was proposed (Strehler, 1986) that mammals with longer life spans obviated the causes of aging of short-lived mammals and therefore age for different reasons. Yet given the large similarities in physiology amongst mammals and their aging phenotype, the most likely scenario is that aging evolved in the primordial mammals and then, in certain species such as the modern long-lived mammals, longevity evolved as a result of a slowing of the basic aging process; but since even the longest-living mammals still age quite dramatically when compared to long-lived reptiles, this implies that they only slowed whatever mechanisms caused aging, not eradicated them, therefore making the causes of aging the same for all mammals independent of life span. *Anthecinus* and some rodents (Finch, 1994, pp. 608 and 616) are at present the only documented cases possible of being exceptions. But since aging has only been described in a fraction of the approximate 4600 species of mammals on the planet, it is possible that some mammalian species have divergent evolution regarding aging. For example, little is known about egg-laying mammals' aging, although, since egg-laying mammals diverged from eutherians 190 million years ago (see Fig. 2), it is not surprising to notice that the Australian spiny anteater has a 50-year life span and presumably a slow rate of aging (Finch, 1994, p. 606).

One hypothesis appears plausible: aging has the same causes in all primates. Although information is scarce regarding fossil records, the evolution of longevity in the first primates began about 60 million years ago (O'Neil, 1999, 2000). This is a good indicator that all primates share the same origin of aging since evolution of longevity and not evolution of aging was taking place.

3.2. How to test this hypothesis?

The ideal scenario to prove this hypothesis and, optimistically, to uncover the causes of mammalian aging would involve comparative biology of the mammalian, reptile, and/or amphibian genomes. Using as models apparently non-aging species, we might then be able to use bioinformatics to map the genes and proteins behind the reptilian secret of longevity. If mammals lost the genes that allow some reptiles to avoid aging, then genes mapped in these reptiles or some amphibians—assuming amphibians possess these genes too—could be used to create transgenic mammals capable of delaying and eventually eradicating aging. Alternatively, we might be able to make knock-ins of mammalian genes involved in whatever deleterious mechanism causes aging. Finally, determining expression profiles of genes theorized to be involved in aging, for example, DNA repair proteins at different ages amongst reptiles and mammals, or even between mammals with different rates of aging, might prove useful in determining what causes aging.

In order to obviate expensive and time consuming DNA sequencing methods, it might be possible to use cellular studies (Austad, 2001) to investigate the reasons behind, for example, reptilian longevity.

Cellular studies in cells from the Galapagos tortoise (Goldstein, 1974) show a large but limited replicative capacity (approximately 110 divisions from fibroblasts extracted from adult animals of unknown age); studies on amphibians demonstrated that cell differentiation does not need to involve loss of genomic totipotency (Gurdon et al., 1975); and the latent period of tissue extracts from frogs is dependant on donor age (Steinhardt, 1986). Unfortunately, cell senescence studies on reptiles have been mostly discarded and progresses made in culture and media conditions could lead to new observations. Information on cell cycle control (e.g. telomere maintenance and telomerase activity), oxidative defenses, stress-response mechanisms, DNA repair, etc., could prove useful to determine the differences in aging between mammals and their ancestry.

Although slowly progressing age-related changes have been reported in amphibians and reptiles (Perez-Campo et al., 1993; Finch, 1994, pp. 143 and 144; Townes-Anderson et al., 1998), there is a very limited amount of data regarding their aging phenotype. Slowly, but noticeable, aging reptiles might be relevant sources of information for they might allow us to find new clues as to the events leading to the evolution of aging from reptiles and possible similarities to human aging. It is probable that amongst the roughly 7000 species of reptiles and 4000 species of amphibians we find aging, even if a slower manifestation of the process found in mammals. For example, we do not know if the causes of death of old reptiles, animals, for example, in captivity, are similar to younger members of the same species.

Performing knock-outs of genes theorized to be involved in human aging in reptiles or amphibians might help clarify which pathways have the higher chances of being right. Werner's syndrome gene homologue in *Xenopus laevis* (Yan et al., 1998) is an excellent candidate, while other DNA repair genes also have a high potential due to their involvement in human progeroid syndromes (Martin and Oshima, 2000). Anti-oxidant enzymes, heat shock proteins, and other proteins involved in the stress response should be studied in these models as stress resistance often correlates with aging rates (Kirkwood and Austad, 2000).

3.3. Implications to gerontological research

If the evolution of aging in mammals was a singular process, then quickly aging evolutionary distant species (e.g. *Drosophila* or *C. elegans*) are dubious models for gerontological research. It can be argued that the mechanisms of aging can always be the same to evolve, i.e. the ones more susceptible to be deleted (Rose, 1991, p. 165). Although certain human aging mechanisms could be common to short-lived species, due to the large differences in physiology, mortality, and body plan between mammals and, for example, insects, it appears unlikely that insects and humans share the same cause of aging. Also, since aging phenotypes are quite diverse in nature, it is logical to assume different causes of aging can exist. Extrapolating, more complex aging phenotypes such as those seen in mammals might therefore be unique. (Obviously, if antagonistic pleiotropy is the reason why mammalian aging evolved then this arguing is useless). Caloric restriction (Weindruch and Walford, 1988), a method of increasing life span common to species such as monkeys (Ramsey et al., 2000), mice, and spiders (Austad, 1989), can be used to show similarities in the aging of many evolutionary distant organisms. It can be argued, however, that caloric restriction, as well as single-gene mutations affecting energy or developmental pathways (Flurkey et al., 2001), delays the entire genetic program, retarding the expression of genes associated with aging, whatever those may be.

If antagonistic pleiotropy is right, then perhaps aging in mammals has the same cause as in birds. Birds also evolved from reptiles; both mammals and birds are the only classes of endoderms, which in turn can lead to increased senses and intellectual capacity and, due to higher metabolic rates, aging as a by-product. It should be noticed that the aging phenotype is less expressed in birds than in mammals of the same size (Holmes and Austad, 1995; Martin, 2000; Holmes et al., 2001), as inferred by the life spans and mortality acceleration curves of most well-studied species. In fact, although oocyte regeneration has never been detected in birds, they might feature non-aging species (Gosden, 1996, pp. 55 and 56). In addition, fossil records do not indicate that present birds evolved from very small animals (Maddison and Maddison, 1998), making birds less receptive to

the evolution of aging. Yet even if low metabolic rates might help explain negligible aging in reptiles, they fail to explain the appearance of reproductive senescence in mammals. Moreover, marsupials, despite having lower metabolic rates, seem to age faster than eutherians of the same size (Austad and Fischer, 1991; Austad, 1997, pp. 88–90), which supports the view that eutherians evolved longevity and developed defense mechanisms not present in marsupials. In birds we witness the opposite (Martin, 2000). So, in conclusion, metabolic rates alone are not behind mammalian aging.

If the hypothesis presented in this paper is correct, then primates, and probably other eutherians too, are obvious choices for studying human aging: primates are effective models because their aging process probably shares its causes with humans; short-lived mammals can lead us to understand why the rate of aging differs between mammals; and long-lived mammals such as humans, whales, elephants, and bats, long-lived for their metabolic rate, can have evolved different mechanisms to retard aging and increase longevity.

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CHAPTER 7: ROLE OF THE TELOMERES IN SIPS

Based on our evolutionary model, we decided to study ageing using human cells. By focusing on human physiology, we avoid having to extrapolate our results from model organisms. Of course that *in vitro* cell culture is not a phenocopy of human ageing, but since most animal models that we advocate are practically impossible to study *in vivo*, we started by working with cellular biology and could not find a better organism to study than Man.

Since telomeres are so important in RS (Wright and Shay, 2001 for arguments), we wanted to investigate their role in SIPS. Our strategy was to employ a cell line (hTERT-BJ1) immortalized with the catalytic subunit of human telomerase.

During our work, we tried to establish the molecular mechanisms responsible for SIPS in both cell lines in hope of determining whether telomeres are relevant or not to SIPS.

7.1. Article 2: Stress-induced premature senescence in BJ and hTERT-BJ1 human foreskin fibroblasts (*FEBS Letters*, volume 523, pages 157-162, 2002)

Our first step was to establish a model of SIPS using hTERT-BJ1 and control BJ cells. We chose to use two stress models: a single 2-hour H_2O_2 stress and five repeated UVB stresses to induce SIPS; we collaborated with Florence Chainiaux in establishing a model for inducing SIPS with UVB (Chainiaux *et al.*, 2002). To determine the subcytotoxic dose of each stressor necessary to induce SIPS, mortality curves were obtained for each cell line and senescent biomarkers were studied: SA β-gal activity, cellular morphology, and [^{3}H]-thymidine incorporation.

In this first article on SIPS, our goal was to establish a working model and have a general view of the mechanisms involved. After we established our model of SIPS, we determined whether telomere length was affected by stress. Since we also sought to elucidate the mechanisms involved, we investigated the effect of SIPS on the protein levels of p53, $p21^{WAF1}$, and $p16^{INK4a}$, as well as on the phosphorylation status of pRb, by Western blot analysis. Lastly, we also determined how stress affects the expression levels of TGF-β1 by reverse transcription-polymerase chain reaction (RT-PCR). Intriguingly, the expression levels of TGF-β1 were not affected by either of the stresses. Since we knew that different cell lines respond to TGF-β1 differently (Kim *et al.*, 1991; Massague, 1998), we assumed that TGF-β1 did not participate in SIPS in BJ cells and so decided not to continue investigating its involvement.

This was the first ever published paper to show that SIPS can occur in normal HDFs immortalized with telomerase. hTERT-BJ1 and parental BJ cells responded to UVB and H_2O_2 stress similarly. Since telomere shortening was only slightly increased in SIPS, our results suggested that mechanisms other than the telomeres must be at work.

Stress-induced premature senescence in BJ and hTERT-BJ1 human foreskin fibroblasts

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Abstract To test the involvement of the telomeres in the senescent phenotype, we used telomerase-immortalized human foreskin fibroblasts (hTERT-BJ1). We exposed hTERT-BJ1 and parental BJ cells to either UVB or H_2O_2 subcytotoxic stress(-es). Both cell lines developed biomarkers of replicative senescence: loss of replicative potential, increase in senescence-associated β-galactosidase activity, typical senescence-like morphology, overexpression of $p21^{WAF-1}$ and $p16^{INK-4a}$, and decreased level of the hyperphosphorylated form of pRb. Telomere shortening was slightly higher under stress for both BJ and hTERT-BJ1 but still much lower than that reported for other cell lines. We conclude that pathways alternative to telomere shortening must cause the appearance of the senescence phenotype.

Key words: Cellular senescence; Fibroblast; Telomere; Telomerase; H_2O_2; UVB

1. Introduction

Telomeres are repeated sequences at the end of chromosomes that shorten, normally by 20–200 bp, with each cell division. Previous research suggested that telomere shortening represents a mechanism for counting cell division triggering cellular senescence [1]. Short telomeres might activate a p53 DNA damage response pathway that in turn leads to growth arrest [2]; cellular senescence can also be induced by the pRb pathway [3]. Telomerase is a reverse-transcriptase enzyme that elongates the telomeres [4]. The catalytic subunit of human telomerase was transfected into normal human fibroblasts. Cell lines were thereby established that failed to reach cellular senescence without transformation [5].

Stress-induced premature senescence (SIPS) is characterized by a cell cycle arrest similar to cellular senescence. SIPS can be triggered by way of single or repeated subcytotoxic stress such as UVB [6] or H_2O_2. After at least 48 h of recovery, the cells begin to display biomarkers of cellular senescence: senescence-associated β-galactosidase (SA β-gal) activity, changes in the expression of several genes, similarities in the regulation of the G1 growth arrest, cellular morphology, etc. (for a review see [7]).

WI-38 fetal lung human diploid fibroblasts (HDFs) kept under 40% O_2 for three population doublings (PDs) undergo SIPS. An accelerated telomere restriction fragment (TRF) shortening of 500 bp per PD is observed [8]. When subcytotoxic H_2O_2 stress or five repeated subcytotoxic *tert*-butylhydroperoxide (t-BHP) stresses are performed on cells during a given PD, respectively, a 322 ± 55 bp and a 381 ± 139 bp TRF shortening are observed during the first PD after stress [9]. HDFs at early PD exposed to 150 μM H_2O_2 once or 75 μM H_2O_2 twice in 2 weeks display biomarkers of senescence. Two treatments with 75 μM H_2O_2 fail to induce significant TRF shortening, suggesting that SIPS can emerge without telomere shortening [10].

In this work, we compared SIPS induced by H_2O_2 or UVB in human diploid BJ fibroblasts expressing telomerase (hTERT-BJ1) and in parental BJ cells to know if major differences would be found in the proportion of cells entering SIPS when telomerase activity is present.

2. Materials and methods

2.1. Cell culture and exposure to UVB and H_2O_2

hTERT-BJ1 HDFs were purchased from Clontech (USA) at 111 PD and stressed around 130–140 PD. BJ HDFs were a kind gift of Dr. E.E. Medrano, Baylor College (USA). HDFs were routinely subcultivated as previously described [11].

Confluent cultures of BJ HDFs at early PD and hTERT-BJ1 HDFs were submitted to five repeated subcytotoxic exposures to UVB stress with one stress per day for 5 days as described previously [6]. Confluent cultures were submitted to a single 2 h exposure to H_2O_2 diluted in medium+10% fetal calf serum (FCS), as was described previously [12]. Control cultures at the same PD followed the same schedule of medium changes without UVB or H_2O_2 treatment.

2.2. Cytotoxicity assays

At 48 h after the (last) stress, cells were washed twice with phosphate buffer saline (PBS) and lysed with NaOH 0.5 N.

To measure cytotoxicity, five exposures to UVB or a single exposure to H_2O_2 stress were performed at increasing doses. Cytotoxicity was measured at 48 h after the (last) stress. The cellular protein content was assayed by the Folin method [13], which has proven to give results similar to those found with the MTT assay and cell counts [6,14,15]. Triplicates were always performed. Results are expressed as mean values ± S.D.

2.3. SA β-gal activity and [^{3}H]thymidine incorporation

At 48 h after the (last) stress, the cells were seeded at a density of 700 cells/cm^2. After 24 h, SA β-gal activity was assessed as described in [16]. The proportion of cells positive for SA β-gal was determined in three samples of 400 cells per dish, each sample in a different dish. Results are expressed as mean values ± S.D.

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Abbreviations: SIPS, stress-induced premature senescence; SA β-gal, senescence-associated β-galactosidase; HDF, human diploid fibroblast; PD, population doubling; TRF, terminal restriction fragment; t-BHP, *tert*-butylhydroperoxide

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At 24 h after the (last) stress, cells were seeded at 10 000 cells/2 cm^2 well. 1 μCi [^{3}H]thymidine (specific activity: 2 Ci/mmol, Du Pont NEN, USA) was added to the culture medium for 48 h. The incorporated radioactivity was quantified by a scintillation counter (Beckman Coulter Inc., USA). The procedure was repeated each day for 4 days after stress. Triplicates were always performed. Results are expressed as mean values ± S.D.

2.4. Telomere length and telomerase activity

Two days after the (last) stress, cells were trypsinized and seeded at a 1:2 ratio. When cells became confluent, genomic DNA was extracted, digested with 25 U *Rsa*I and *Hin*fI (Pharmingen, USA), electrophoresed, transferred to a nylon membrane, pre-hybridized and hybridized with a 51-mer biotinylated telomere probe using the reagents and buffers of the Telomere Length Assay kit (#559838, Pharmingen, San Diego, CA, USA), as used previously [9]. The mean terminal restriction fragment (TRF) length was calculated for each sample by integrating the signal density above background over the entire TRF distribution as a function of TRF length, using the formula $L = \Sigma(OD_i \times L_i)/\Sigma(OD_i)$, where OD_i and L_i are respectively the signal intensity and TRF length at position i on the gel image, as described in the manufacturer's recommendations. We also determined telomere length before the (first) stress. Results are expressed as mean values ± S.D. on three experiments. Telomerase activity was studied by TRAP assay (Intergen Inc., USA) following the manufacturer's recommendations.

2.5. Western blot detection of p53, p21^{WAF-1}, p16^{INK-4a} and pRb

The cells were washed twice with ice-cold PBS and protein extraction was conducted as previously described [12]. Samples of 20 μg protein were electrophoresed and transferred overnight on Immobilon-P membrane (Millipore, Bedford, MA, USA). Each protein was detected with its specific antibody: anti-p16, anti-p21, anti-Rb (Santa Cruz, Germany) and anti-p53 (Pharmingen). After incubation with horseradish peroxidase-linked secondary antibody, the bands were visualized after incubation with chemoluminescent substrates using the ECL detection kit (Pharmacia, Belgium). Results are expressed as mean values ± S.D. on three experiments.

3. Results

3.1. Cytotoxicity after UVB irradiation or H_2O_2 stress

The results were expressed as percentages of the controls, which were subjected to the same conditions for the same period of time without being subjected to H_2O_2 or UVB. As expected, the cytotoxicity increased with the UVB or H_2O_2 doses (Fig. 1A,B). BJ and hTERT-BJ1 cells behaved similarly. From day 0 to day 3 (= 48 h after H_2O_2 stress) and day 7 (= 48 h after the last UVB stress), we found that the cellular protein content of the controls increased roughly by, respectively, 20 and 40%. Thus an apparent decrease of 20 or 40% of cellular protein shown at 48 h after stress with respectively H_2O_2 or UVB corresponds to absence of growth rather than cell death. These results suggested to use the doses of 1.2 mM H_2O_2 and 600 mJ/cm^2 in our experimental conditions and to consider them as subcytotoxic doses.

3.2. SA β-gal activity, cell morphology and cellular proliferation

SA β-gal activity was described to appear in replicative senescence and SIPS [16]. After a single H_2O_2 stress at 1.2 mM H_2O_2, the percentage of SA β-gal positive cells increased by 15–20% in both BJ and hTERT-BJ1 cells (Fig. 2). When hTERT-BJ1 cells were exposed to H_2O_2 at a cytotoxic concentration (1.5 mM) the percentage of positive cells increased only by 7% (not shown). After five UVB stresses, the percentage of cells positive for SA β-gal activity increased by 30–35% at 600 and 800 mJ/cm^2 UVB for BJ cells and reached similar values at 500 and 800 mJ/cm^2 UVB for hTERT-BJ1 cells (Fig. 2). An appreciation of cellular morphology shows that after a H_2O_2 stress, both BJ and hTERT-BJ1 cells develop a higher incidence of abnormal morphology, resembling a senescent morphology. The differences between controls and stressed cells are not so intense following five UVB stresses.

Subcytotoxic stress with agents such as H_2O_2, t-BHP or UVB triggers irreversible growth arrest of a large proportion of a population [6,9,17]. In this work we tested whether cells transfected with hTERT would show a different behavior after being stressed with UVB or H_2O_2 as far as [^{3}H]thymidine incorporation is concerned. After a single H_2O_2 stress, the level of incorporation fell by 50% in BJ and hTERT-BJ1 HDFs, indicating that only about 50% of the cells were still able to divide when compared to non-stressed controls, from day 3 to day 7 after stress (on the graph, day 1 indicates the first day of measurement of incorporation corresponding to 72 h after stress) (Fig. 3). After five consecutive UVB stresses, the difference was smaller, being respectively 20–25% from day 3 to day 7 after the last stress for BJ and for hTERT-BJ1 cells (Fig. 3). The time-dependent decrease observed in hTERT-BJ1 cells, stressed or unstressed, was highly reproducible and will be considered in Section 4.

3.3. Telomere shortening

A very low and similar TRF shortening occurred in both BJ and hTERT-BJ1 cells without stress: 37 ± 18 bp/PD and 43 ± 13 bp/PD, respectively. These results are within those described earlier [18,19]. After H_2O_2 stress, TRF shortening was multiplied by two in BJ cells and by four in hTERT-BJ1

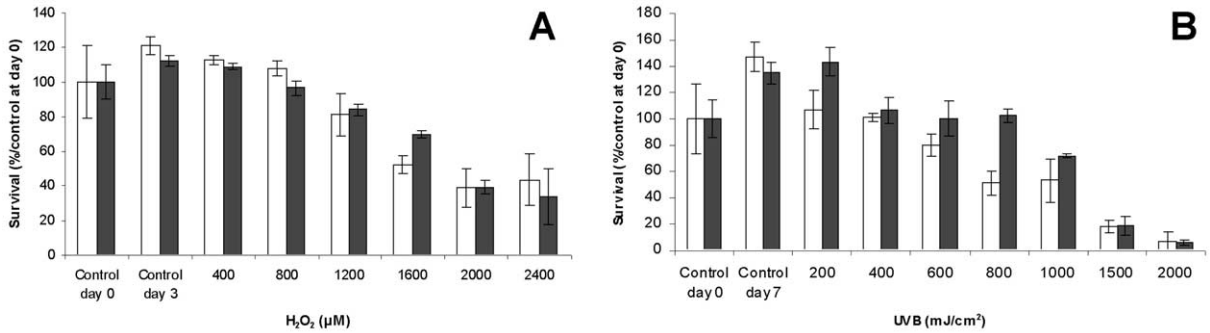

Fig. 1. Cytotoxicity of a single exposure to H_2O_2 (A) or five repeated exposures to UVB (B) with one stress per day in BJ (white columns) and hTERT-BJ1 (gray columns) HDFs. The results are expressed as percentages of the values found in control cells at 48 h after the (last) stress. Results are given as mean values ± S.D. from three independent experiments.

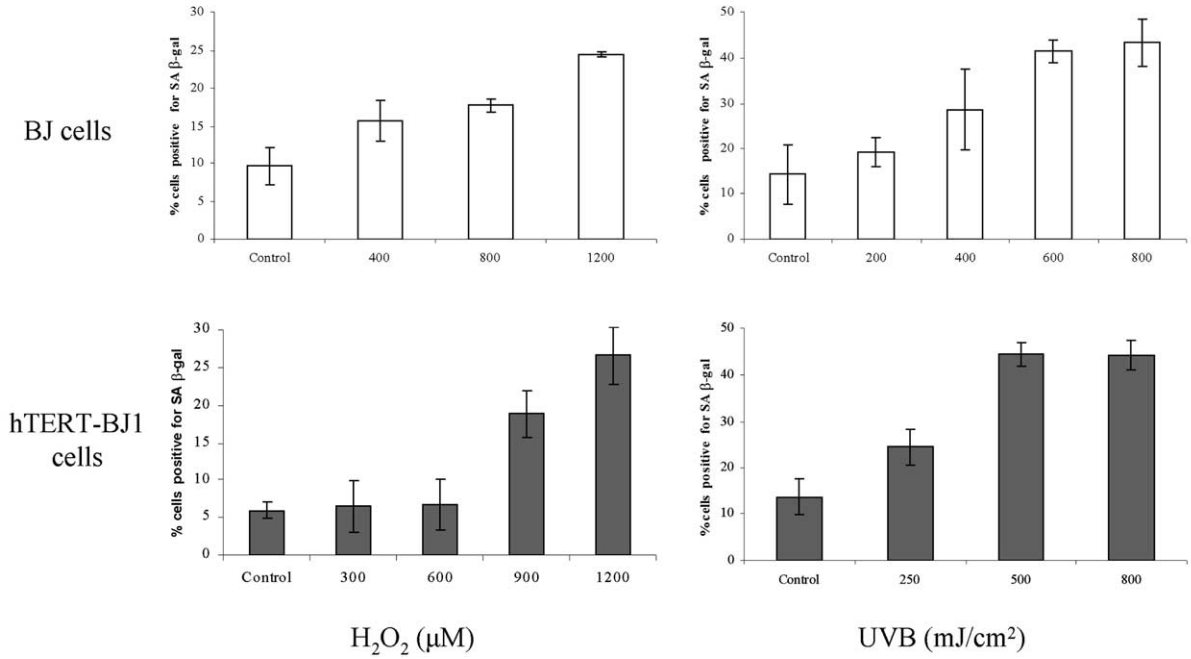

Fig. 2. Effects of a single H_2O_2 stress or five repeated UVB exposures on the proportion of BJ and hTERT-BJ1 HDFs positive for SA β-gal activity. Results represent the proportion of cells positive for SA β-gal. The results are presented as mean values ± S.D. from three independent experiments.

cells, indicating that H_2O_2 stress affected telomere length (Fig. 4A). We did not explain that difference between the two types of cells. After repeated UVB stresses, BJ cells underwent a TRF shortening of 70 ± 27 bp, while TRF shortening in hTERT-BJ1 cells was 38 ± 30 bp. It might be considered that about 25–30% of the cells do not resume mitosis after the stress, from the data obtained with [^{3}H]thymidine incorporation and SA β-gal histochemistry. Calculations of expected TRF shortening due to compensatory cycling of the fraction of cells recovering mitotic capability suggest that the limited shortening observed cannot be accounted for by the compensatory cycling and is also supposed to be caused by DNA damage.

3.4. Telomerase activity

Although we cannot discard minor telomerase activity fluctuations, our results suggest that telomerase activity is not as affected by the stress as when SIPS is established (Fig. 4B). Indeed, we detected similar telomerase activity before and after each type of stress in hTERT-BJ1 cells. No telomerase activity was detected in BJ cells. These findings confirm previous results obtained in different experimental conditions [19].

3.5. Expression level of p53, $p21^{WAF-1}$, $p16^{INK-4a}$ and phosphorylation level of Rb

It was shown that p53 is overexpressed by IMR-90 HDFs after subcytotoxic H_2O_2 stress and comes down to basal level within 44 h after the stress [17]. Only minimal overexpressions of p53 were found in BJ cells at 72 h after H_2O_2 stress (about 20%) and after UVB stress (about 40% overexpression). No overexpression of p53 was found in hTERT-BJ1 cells at 72 h

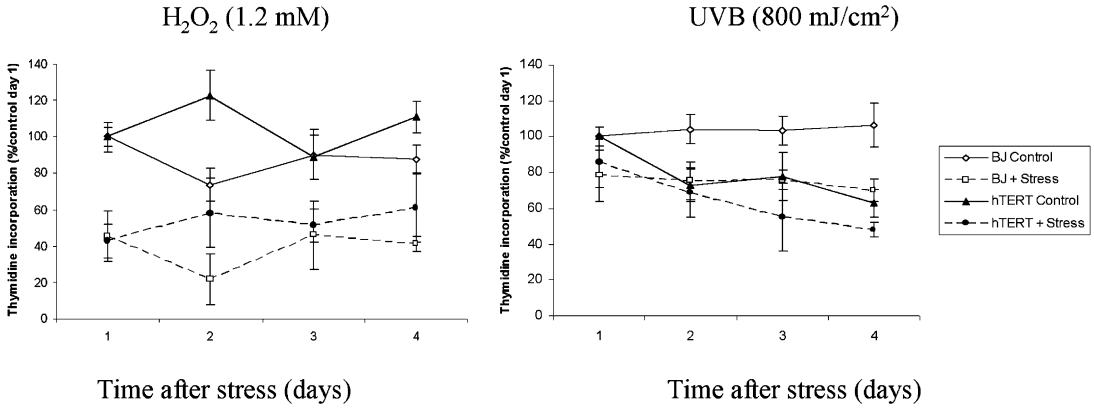

Fig. 3. Estimation of the proliferative potential of BJ and hTERT-BJ1 HDFs exposed to a single H_2O_2 stress or five consecutive exposures to UVB by measurement of the incorporation of [^{3}H]thymidine into DNA between day 1 and day 4 after stress. The results obtained are expressed as percentages of the cpm incorporated by the control cells. The results represent the mean values ± S.D. from three independent experiments.

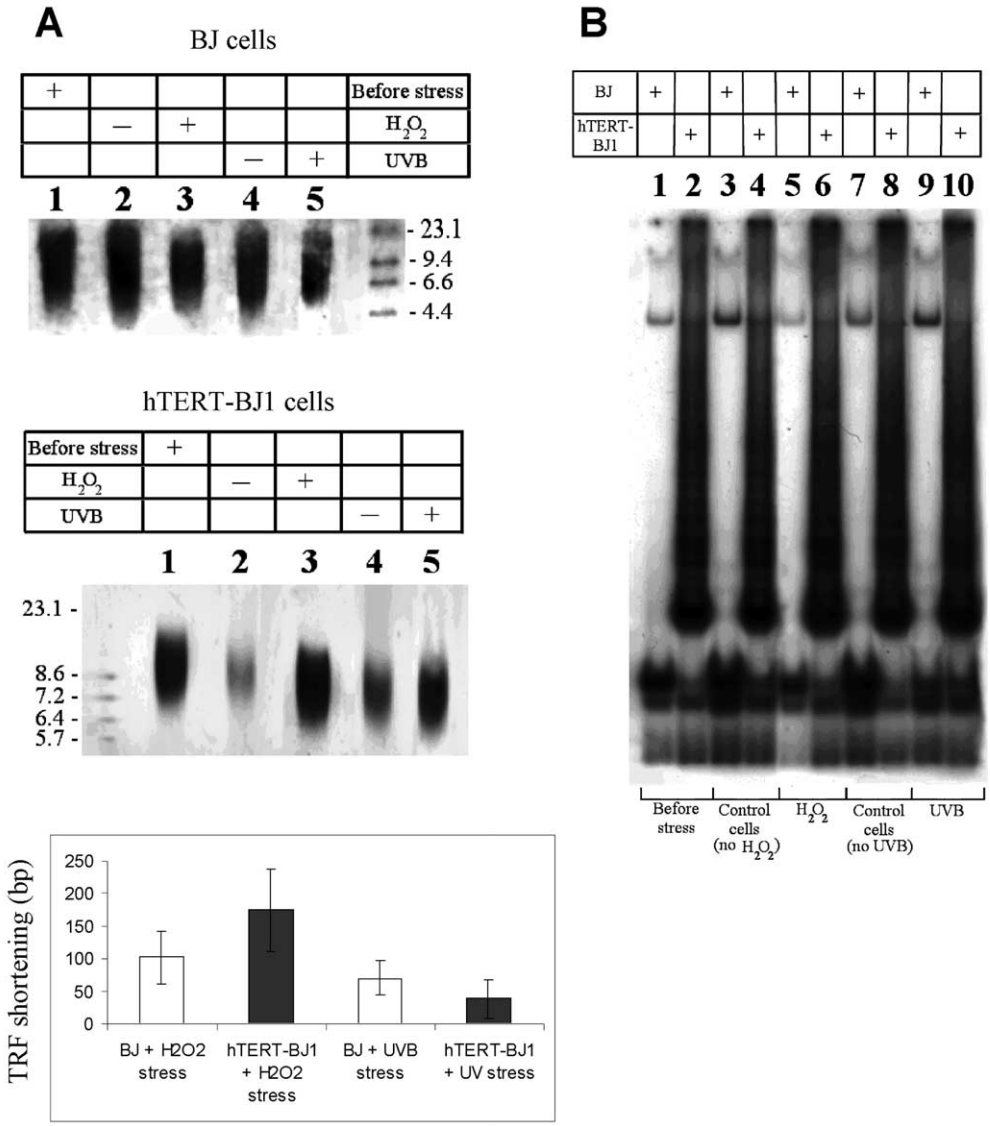

Fig. 4. A: Effect of a single H_2O_2 or five UVB exposures on telomere shortening in BJ and hTERT-BJ1 HDFs. Lane 1: cells at confluence before the stress. Lane 2: control cells for H_2O_2 experiment. Lane 3: cells exposed to H_2O_2. Lane 4: control cells for UVB experiment. Lane 5: cells exposed to UVB. Telomere shortening of stressed cells was measured by comparing telomere length of stressed cells to controls. Controls correspond to HDFs submitted to the same culture conditions as the stressed cells but without any H_2O_2 or UVB. The results represent the mean values ± S.D. from at least three independent experiments. B: No effect of a single H_2O_2 or five UVB exposures on telomerase activity. Lane 1: BJ cells before the experiment. Lane 2: hTERT-BJ1 cells before the stress. Lane 3: control BJ cells for H_2O_2. Lane 4: control hTERT-BJ1 cells for H_2O_2. Lane 5: BJ cells exposed to H_2O_2. Lane 6: hTERT-BJ1 cells exposed to H_2O_2. Lane 7: control BJ cells for UVB. Lane 8: control hTERT-BJ1 cells for UVB. Lane 9: BJ cells exposed to UVB. Lane 10: hTERT-BJ1 exposed to UVB.

after both types of stress (Fig. 5A.1). This suggests that p53 remains overexpressed a little longer in BJ cells compared to IMR-90 cells and the presence of telomerase activity blocks this overexpression.

$p21^{WAF-1}$ is overexpressed at 72 h after five repeated t-BHP stresses in WI-38 HDFs [14] and after a single H_2O_2 stress in IMR-90 HDFs [15,17]. A 2.1- and 1.8-fold overexpression of $p21^{WAF-1}$ was found at 72 h after subcytotoxic H_2O_2 stress and five repeated UVB stresses on BJ cells, respectively (Fig. 5A.2). In hTERT-BJ1 cells, a 1.8- and 1.5-fold overexpression of $p21^{WAF-1}$ protein was found at 72 h after, respectively, H_2O_2 or UVB stress. Measurements of the DNA binding capability of p53 would give information on the dependence of this overexpression of $p21^{WAF-1}$ toward p53. No difference of $p16^{INK-4a}$ protein level was observed in BJ cells exposed to H_2O_2 and in hTERT-BJ1 cells exposed to H_2O_2 or UVB (Fig. 5A.3). A limited 35% overexpression was observed in BJ cells exposed to UVB. A similar change in the phosphorylation status of pRb was observed after stress in all four situations (UVB and H_2O_2 in BJ and hTERT-BJ1 cells) (Fig. 5B).

4. Discussion

SIPS has previously been demonstrated to occur after subcytotoxic stress with, for example, H_2O_2 in IMR-90 HDFs, t-BHP and hyperoxia in WI-38 HDFs (for a review see [7]) and in FS skin HDFs exposed to UVB [6]. BJ HDFs are extremely resistant to hyperoxia and H_2O_2 [19]. This work also shows the remarkable resistance of these cells to H_2O_2 and UVB, with respective subcytotoxic doses of 1.2 mM and

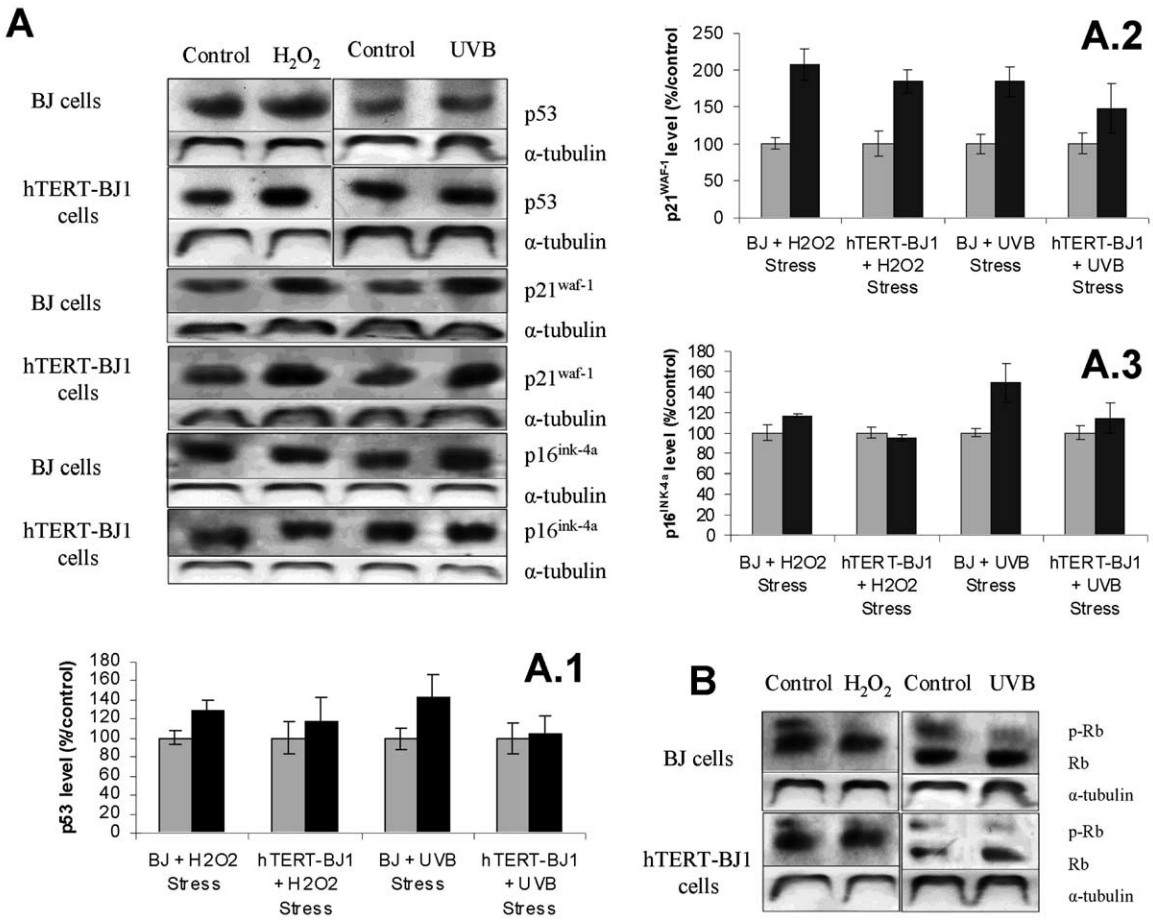

Fig. 5. A: Western blots and quantifications of the protein level of p53 (A.1), $p21^{WAF-1}$ (A.2), and $p16^{INK-4a}$ (A.3) when BJ and hTERT-BJ1 cells are exposed once to H_2O_2 or five times to UVB. B: Phosphorylation status of the Rb protein at 72 h after stress. P-Rb stands for phosphorylated Rb; Rb for hypophosphorylated Rb. The results represent the mean values ± S.D. from three independent experiments.

0.15 mM H_2O_2 in BJ and IMR-90 HDFs [12], and respective subcytotoxic doses of 600 and 500 mJ/cm^2 UVB in BJ and FS HDFs [6], these two strains being derived from the skin.

The growth of BJ HDFs and their telomerase positive derivates was only slightly reduced under hyperoxia. A high resistance of BJ cells to oxidative damage is known [19]. We found a lower proportion of BJ cells to enter SIPS after subcytotoxic H_2O_2 stress. Indeed, the percentage of SA β-gal positive cells after exposure of two strains of fetal lung HDFs, IMR-90 and WI-38, to, respectively, 150 and 160 μM H_2O_2 was around 55% [9,12]. However, similar results were obtained after UVB stress at the respective subcytotoxic doses of 600 and 500 mJ/cm^2 in BJ and FS HDFs [6].

Both BJ and hTERT-BJ1 cells showed a decrease in proliferative capability on the long term after H_2O_2. The level of [^{3}H]thymidine incorporation was not much different in the BJ and hTERT-BJ1 cells after UVB. There might be a lag time before unstressed BJ and hTERT-BJ1 cells resume mitosis after the many days of confluency occurring between and after the UVB stresses (7–11 days before [^{3}H]thymidine incorporation finishes). This could explain the small difference of thymidine incorporation between the UVB-stressed and non-stressed BJ and hTERT-BJ1 HDFs.

After a period of extension of telomere length following ectopic expression of telomerase, telomere length starts to fall slowly over time, reaching a length similar to the parental cells [19], as confirmed in this work. The remaining low telomerase activity appears to stabilize predominantly the shortest telomeres, allowing the growth of these cells despite the average telomere length becoming eventually shorter than in the parental cells at senescence [20]. Therefore, these cells were ideal to test whether SIPS could still appear despite the presence of an hTERT activity that would maintain the telomeres at a subcritical length, thereby allowing the maintenance of the cell cycle.

The limited telomere shortening after stress was found to be about two and four times higher after H_2O_2 stress than in control cells, in BJ and hTERT-BJ1 cells, respectively. This is intriguing since telomerase remained active in the stressed cells and control hTERT-BJ1 cells. It could be that this TRF shortening affected those telomeres which were at a subcritical length, thereby triggering growth arrest. After the UVB stress, the presence of telomerase activity seems to have offered some protection against any further telomere shortening. Anyway, these different shortenings did not lead to a different percentage of cells in UVB-induced SIPS in BJ or hTERT-BJ1 cells. It might, however, have affected the kinetics of recovery of the proportion of the cell population not in SIPS. This result can be correlated with the slight increase in $p16^{INK-4a}$ level after UVB only in BJ cells.

It is possible to induce the senescence of HeLa cells, which have short telomeres, by repressing the human papillomavirus

type 18 E6 and E7 genes, resulting in reactivation of the dormant p53 and pRb tumor repressor pathway. Stable clones of HeLa cells that express hTERT have elevated telomerase activity and extended telomeres. These clones gave an identical response when the E6 and E7 proteins were repressed: growth arrest, SA β-gal activity, altered morphology and increased autofluorescence. Therefore, HeLa senescence induced by these means was not triggered by short telomeres [21]. pRb has been shown not only to be involved in the control of the cell cycle but also in the appearance of different biomarkers of senescence after H_2O_2 stress in IMR-90 HDFs (SA β-gal activity, altered morphology, overexpression of fibronectin, osteonectin, apolipoprotein J) [12,22]. In this cell strain, overexpression of transforming growth factor-β1 (TGF-β1) starting at 24 h after subcytotoxic H_2O_2 stress triggered the appearance of these biomarkers. TGF-β1 overexpression disappeared when E7 was stably expressed in these cells [12]. However, we failed to find any increase in TGF-β1 after UVB or H_2O_2 stress in BJ or hTERT-BJ1 cells (results not shown).

H_2O_2 might have modified the level of the telomeric DNA binding proteins TRF-1 or TRF-2. It was recently shown that overexpression of TRF-2 protects critically short telomeres from fusion and represses chromosome-end fusions in presenescent HDFs, even if accelerated telomere shortening is observed [23]. UVB might not affect the TRF-2 expression level. In addition, it was already known that the introduction of TTAGGG oligonucleotides into HDFs induces a p53- and $p21^{WAF-1}$-dependent long-term growth inhibition [24]. Binding of TRF-2 protein by these oligonucleotides might also explain these results.

In conclusion, this study shows that, besides telomere shortening and TGF-β1 overexpression, other mechanisms might exist which can trigger SIPS.

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CHAPTER 8: GENE EXPRESSION IN SIPS

From a theoretical perspective, having the human genome sequence means having the digital code from which all processes arise, including ageing and cellular senescence. Unfortunately, deciphering that code is a monumental task. The development of computer applications to make sense of the flood of data invading the life science is crucial (reviewed in Luscombe *et al.*, 2001). Moreover, a combination of experimental and computational tools will be necessary to understand the genome and how it governs the complex human physiology. One of the modern high-throughput techniques is the DNA microarray to study gene expression. We wanted to employ DNA microarrays to study SIPS and develop methodologies to study ageing at a genomic level.

8.1. Article 3: All roads lead to Rome: How gene expression networks reorganize in premature senescence of human skin fibroblasts expressing or not telomerase (submitted for publication)

Once with the model established, we wanted to have a more detailed view of SIPS in BJ cells. Since the TGF-β1 and telomere pathways did not appear to be involved in our model, we wanted to find which pathways were causing SIPS. In association with Eppendorf Array Technologies (Namur, Belgium), we employed the low-density DNA microarray DualChip Human General to study gene expression patterns before and after a single H_2O_2 stress. The DualChip Human General represents 202 pre-selected genes of general interest in cell biology. We decided to focus our study on SIPS induced by H_2O_2 since as judged by the biomarkers of senescence, such as [^{3}H]-thymidine incorporation, the senescent phenotype is more pronounced due to a single H_2O_2 stress than to five repeated UVB stresses.

In addition to studying gene expression patterns, we also determined the temporal resolution of the DNA-binding activity of several transcription factors. By determining transcription factor activity in conjunction with gene expression we hoped to understand the underlying mechanisms of SIPS in our model.

Our gene expression results suggested a rearrangement of gene expression patterns with SIPS in both hTERT-BJ1 and BJ HDFs. Also taking into account our results from article 2, we defined a model where p53 and $p21^{WAF1}$ appear as crucial components of SIPS. Telomeres/telomerase did not appear to play a critical role in SIPS and telomerase did not protect against SIPS. Importantly, our results also suggested that telomerase is unlikely to become a

useful anti-ageing therapy since it changes the normal cellular functions and may promote tumorogenesis.

Although we focused on SIPS of hTERT-BJ1 and BJ cells induced by H_2O_2, we also studied a cell line--AG00780--derived from a patient with WS that we obtained from the American Type Culture Collection (Rockville, Maryland, USA). We attempted to establish a model of SIPS using WS fibroblasts, which was not satisfactory. Even so, we suggested that gene expression patterns in WS fibroblasts are more closely related to old rather than young BJ HDFs.

All roads lead to Rome: How gene expression networks reorganize in premature senescence of human skin fibroblasts expressing or not telomerase

Keywords: aging, cellular senescence, telomeres, H_2O_2

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Abbreviations: bp, base pairs; HDFs, human diploid fibroblasts; PDs, population doublings; RS, replicative senescence; hTERT, catalytic subunit of human telomerase; RS, replicative senescence; SIPS, stress-induced premature senescence; WS, Werner's syndrome; SA βgal, senescence-associated; FCS, fetal calf serum.

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Abstract

We compared the DNA-binding activity of transcription factors and gene expression patterns in BJ human diploid fibroblasts (HDFs) expressing or not telomerase (hTERT) in stress-induced premature senescence (SIPS). Senescent BJ cells and fibroblasts obtained from a patient with Werner's syndrome (WS), which feature defects in DNA repair, were also studied.

H_2O_2-induced SIPS modulated gene expression in both BJ and hTERT-BJ1 cells. Increased $p21^{WAF-1}$ mRNA level was amongst the common gene expression changes in BJ and hTERT-BJ1 cells induced by SIPS. Both cell lines also displayed sustained DNA-binding activity of p53. Telomerase expression markedly changed gene expression in non-stressful conditions. Expression patterns of senescent BJ cells partially overlapped those of BJ and hTERT-BJ1 cells in SIPS. Gene expression patterns of WS fibroblasts were closer to senescent BJ cells than to BJ cells at early population doublings whereas we failed to induce SIPS in WS fibroblasts.

Our results indicate that similar mechanisms involving $p21^{WAF-1}$ and probably p53 are at work in BJ and hTERT-BJ1 HDFs under H_2O_2-induced SIPS, suggesting that generalized DNA damage rather than telomere length/telomerase plays a crucial role in H_2O_2-induced SIPS. We propose that H_2O_2-induced SIPS involves a rearrangement of proliferative and apoptotic pathways. The marked changes in gene expression induced by telomerase suggest that apart from immortalization of HDFs, telomerase also alters the normal cellular functions but does not protect against SIPS.

1. Introduction

Normal human diploid fibroblasts (HDFs) stop dividing after a certain number of population doublings (PDs) *in vitro* (Hayflick and Moorhead 1961). This phenomenon is termed replicative senescence (RS). Telomere shortening observed at each cell division eventually leads telomeres to critical lengths. Critically short telomeres are probably recognized as DNA damage and activate p53, which triggers growth arrest through the overexpression of cyclin-dependent kinase inhibitors such as $p21^{WAF-1}$. $p21^{WAF-1}$ in turn inhibits the phosphorylation of the retinoblastoma protein (Vaziri and Benchimol, 1996). Telomerase is a reverse-transcriptase enzyme that elongates the telomeres. HDFs transfected with the catalytic subunit of human telomerase (hTERT), although they are not transformed, do not display RS when considered as cell populations (Bodnar et al., 1998). Recent data, however, indicates that high levels of telomerase expression could favor RS of a small fraction of the cell population of HDFs (Gorbunova et al., 2003).

Stress-induced premature senescence (SIPS) establishes several days after exposure of HDFs to subcytotoxic concentrations of numerous types of oxidants and DNA-damaging agents such as hydrogen peroxide (H_2O_2) or UV radiation. HDFs in SIPS display features common with RS: a senescent morphology, senescence-associated β-galatosidase (SA βgal) activity, growth arrest in the G1 phase of the cell cycle, etc. (Chen et al., 1998, 2000). Oxidative stress can increase the shortening of telomeres in HDFs. WI-38 HDFs exposed to 40% hyperoxia undergo a mean telomere shortening of 500 bp per PD, compared to the 90 bp/PD normal shortening observed under 20% atmospheric O_2. Such accelerated shortening rapidly leads the telomeres to their critical length, triggering irreversible growth arrest of the HDFs (von Zglinicki et al., 1995). Yet H_2O_2 can induce SIPS without critical shortening of the telomere length. After exposure to subcytotoxic H_2O_2 stress, BJ foreskin HDFs expressing hTERT display features of senescent cells at levels comparable to the wild type BJ HDFs exposed to the same concentration of H_2O_2. Only a slight telomere shortening is observed in both BJ and hTERT-BJ1 HDFs (de Magalhaes et al., 2002; Gorbunova et al., 2002) and so the role of the telomeres in SIPS remains unclear.

Werner's syndrome (WS) is a human autosomal recessive disease caused by a mutation in the Werner protein (WRN), a RecQ helicase. The pathology of WS affects multiple organs and resembles accelerated aging, including skin changes, gray hair, and increased cancer incidence. The WRN protein appears to be related to DNA repair since it has exonuclease activity. Fibroblasts taken from patients with WS have a shorter proliferative lifespan, an extended S phase, and display genomic instability. WS fibroblasts have an increased mean telomere loss, are hypersensitive to 4-nitroquinoline 1-oxide, and sensitive to ionizing radiation,

but not to other DNA-damaging agents such as UV (reviewed by Bohr et al., 2002). Recently, telomerase positive tumor cells expressing a dominant-negative mutation of the WRN protein showed an increased rate of telomere loss and chromosome fusions, but no detectable changes in average telomere length. The WRN protein also appeared to downregulate telomerase (Bai and Murnane, 2003).

We used a low-density DNA array representing genes of general interest in cell biology to characterize gene expression of BJ and hTERT-BJ1 HDFs in H_2O_2-induced SIPS. Our interest was to identify changes in gene expression in BJ cells in SIPS and to know whether similar changes exist in BJ cells expressing hTERT. In contrast to studying the effects of hTERT, a protein that repairs the DNA, we investigated if we could induce SIPS by H_2O_2 in WS fibroblasts, which have increased DNA damage, and study gene expression patterns in WS fibroblasts. Furthermore, we studied gene expression in senescent BJ HDFs. The excellent reproducibility and validity of the data obtained with low-density DNA arrays technically identical to those employed in the present report was previously demonstrated in terms of chemistry of covalent binding of DNA on activated glass support, optimized length and sequence, hybridization, and statistical analysis (de Longueville et al., 2002). Lastly, and in order to help understand the changes in gene expression, we studied the DNA-binding activity of several transcription factors.

2. Materials and Methods

2.1. Cell culture, induction of SIPS by H_2O_2, isolation of mRNA

hTERT-BJ1 HDFs (Clontech, Palo Alto, CA, USA) at 111 PD were exposed to H_2O_2 between PD 130 and 150. BJ HDFs were a kind gift from Dr. E.E. Medrano, Baylor College, Houston, TX, USA and were stressed between PD 20 and 35. Previous reports indicated that the only consequence of hTERT transfection was immortalization (Bodnar et al., 1998; Jiang et al., 1999; Morales et al., 1999). Both BJ and hTERT-BJ1 cells were cultivated in DMEM medium + 10% fetal calf serum (FCS) under the classical conditions previously described (Hayflick and Moorhead, 1961). H_2O_2 at 1,200 μM diluted in medium with 10% serum for 2 hrs was previously shown to be subcytotoxic and trigger SIPS in both BJ cell lines (de Magalhaes et al., 2002). Control cultures at the same PD followed the same schedule of medium changes without exposure to exogenous H_2O_2. At 72 hrs after stress, mRNA was isolated (FastTrack 2.0 mRNA isolation kit, Invitrogen, Carlsbad, CA, USA). Seventy-two hrs after subcytotoxic H_2O_2 stress is an optimal time to study H_2O_2-induced SIPS at least in WI-38, IMR-90, BJ, and hTERT-BJ1 HDFs (Frippiat et al., 2001; de Magalhaes et al., 2002; Gorbunova et al., 2002). AG00780 WS

dermal fibroblasts were obtained from the American Type Culture Collection (Rockville, MD, USA) and were subcultivated in BME + 10% FCS. We exposed confluent cultures at PD 16 to different concentrations of H_2O_2 diluted in medium plus FCS for 2 hrs and determined the cytotoxicity 48 hrs after by measuring lactate dehydrogenase release (Cytotoxicity Detection Kit (#1644793), Roche, Basel, Switzerland). SA βgal activity was detected as described previously (Dimri et al. 1995).

2.2. Low-density DNA array design, synthesis of labeled DNA, and hybridization conditions

The DualChip Human General design (Eppendorf, Hamburg, Germany) is based on a system with two arrays per glass slide and three sub-arrays per array. The array represents a range of 202 genes involved in basic cellular processes such as metabolism, apoptosis, cell cycle, stress response, pro-inflammatory state and transcription (Supplemental Data). The sequences of the DNA covalently linked to the glass slide were carefully chosen by sequence comparison and it was checked experimentally that no cross-hybridization takes place. Several positive and negative hybridization controls plus detection controls were spotted on the array in order to control the reliability of the experimental data. 0.5 μg of each sample of mRNA was retrotranscribed into DNA according to the manufacturer's instructions. Three synthetic poly(A)+tailed RNA standards were spiked at three different amounts (10 ng, 1 ng and 0.1 ng per reaction) into the purified mRNA as required by the array kit (Eppendorf, Hamburg, Germany). Normalization of data was possible thanks to three internal standard controls described above and eight housekeeping genes. Triplicates from three independent experiments were performed, meaning hybridizations on nine sub-arrays. The DualChip Human General hybridization was carried out according to the manufacturer's instructions as reported (de Longueville et al., 2002). Detection was performed using a Cy3-conjugated IgG anti-biotin (Jackson Immuno Research laboratories, West Grove, PA, USA).

2.3. Imaging, statistical analysis, and clustering

Fluorescence of the hybridized arrays was scanned using the Packard ScanArray (PerkinElmer, Boston, MA, USA) at a resolution of 10 μm. To maximize the dynamic range of detection, the same arrays were scanned at different photomultiplier gains for quantifying both the high- and low-copy expressed genes. The scanned 16-bit images were imported into the ImaGene 4.1 software (BioDiscovery, Los Angeles, CA, USA) to quantify the signal intensities. The fluorescent intensity of each DNA spot (average of intensity of each pixel present within the spot) was calculated using local mean background subtraction. A signal was accepted if the

average intensity after background subtraction was at least 2.5-fold higher than their local background. The three intensity values of the triplicate DNA spots were averaged and used to calculate the intensity ratio between the reference and the test samples.

The data were normalized in two steps. First, the values were corrected using a factor calculated from the intensity ratios of the internal standards in the references and test samples. The presence of the three internal standards probes at two different locations of the array allowed a measurement of local background and evaluation of the array homogeneity, which is considered in the normalization. However, since the internal standard control does not take into account the purity and quality of the mRNA, a second step of normalization was performed based on expression levels of the housekeeping genes. This process involves calculating the average intensity for a set of housekeeping genes. The variance of the normalized set of housekeeping genes is used to generate an estimate of expected variance, leading to a predicted confidence interval for testing the significance of the ratios obtained. Ratios outside the 95% confidence interval were determined to be significantly different (reviewed by de Longueville et al., 2002).

2.4. ELISA kits for detection of transcription factor DNA-binding activity

Nuclear extracts were obtained from BJ and hTERT-BJ1 cells exposed or not to a single H_2O_2 stress of 1,200 μM at 2, 6, 24, 48, and 72 hrs after the stress. We then determined the DNA-binding activity of p53, HIF-1, CREB, AP-1, NF-κB, ATF-2, PPARg, and NFAT using the TransAM kits (ActiveMotif, San Diego, CA, USA), according to manufacturer's instructions. Triplicates were always performed from three independent experiments.

3. Results

3.1. Gene expression varies in H_2O_2-induced SIPS in BJ ± ectopic hTERT HDFs

BJ and hTERT-BJ1 human diploid skin fibroblasts were exposed to a 2h-stress with H_2O_2 at 1,200 μM, which represented subcytotoxic conditions. At 72 hrs after stress, both cell lines displayed a large increase in the proportion of the SA βgal positive cells, a sustained decrease in incorporation of [^{3}H]-thymidine into their DNA, and $p21^{WAF-1}$ overexpression (de Magalhaes et al., 2002). The low-density DNA array DualChip Human General was used to study the mRNA levels of 202 pre-selected genes (Supplemental Data, Fig. 1) in BJ and hTERT-BJ1 HDFs in H_2O_2-induced SIPS. At 72 hrs after H_2O_2 stress, 21 genes were found to be differentially expressed in BJ cells, with 14 genes displaying an increased and 7 genes a decreased mRNA steady-state level. In hTERT-BJ1 cells in H_2O_2-induced SIPS, 9 genes displayed an increased

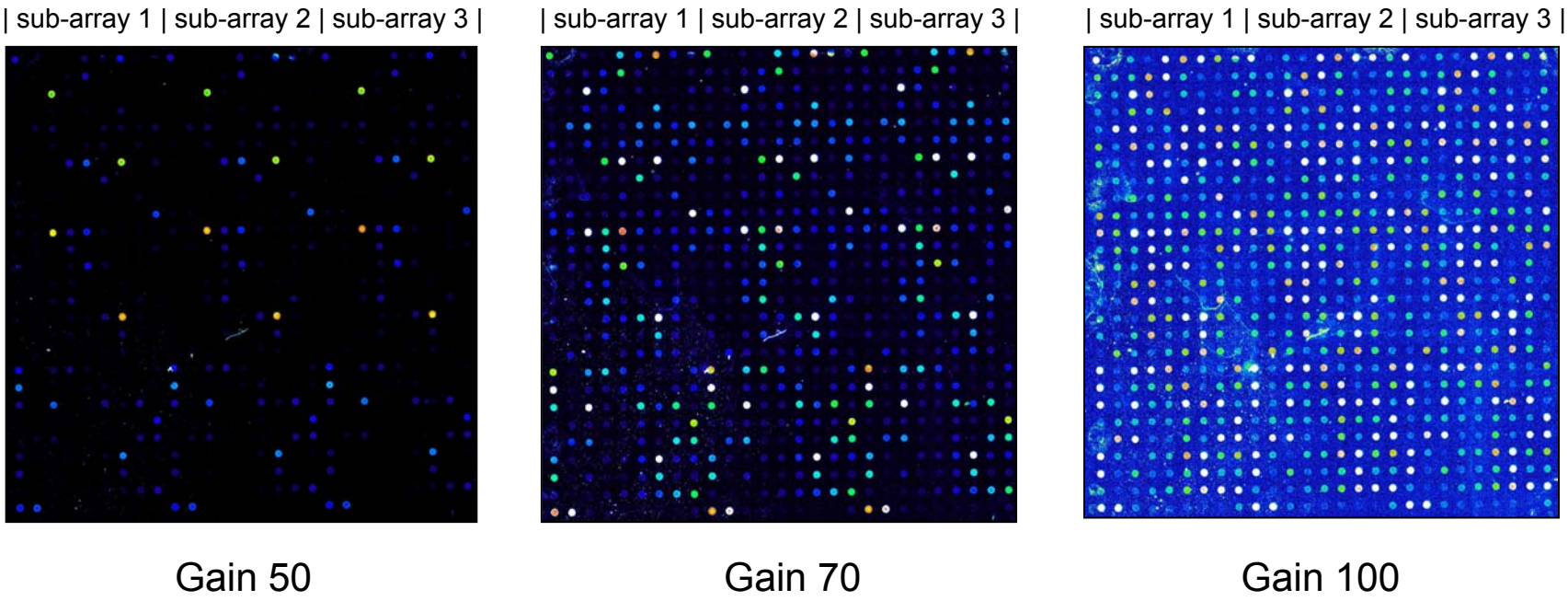


Figure 1: Representative example of images obtained with DualChip scanned at different photomultiplier gains. Each DualChip features triplicate spots (sub-arrays) on each array. Three DualChip were used in each experiment.

while 7 genes displayed a decreased mRNA steady-state level. Thirty-four genes were differentially expressed in BJ cells at late PDs when compared to young BJ cells. Lastly, 54 genes were also differentially expressed in hTERT-BJ1 compared to BJ cells (Table 1). The correspondence between real-time RT-PCR and this array's data has been checked for several differentially expressed genes including: APOJ, FN1, MMP-1, MMP-2, p21, and SM22. Other genes such as FOS, ON, and p53 were also verified though these were not differentially expressed in our experimental conditions. Although mRNA and protein levels may not always correlate, changes in mRNA generally correspond to changes at the protein level. Our gene expression results yield an insight at the regulatory level but further analysis of protein or enzymatic activities is necessary to understand functional changes in these pathways.

3.2. Changes in gene expression triggered by hTERT

The overexpression of CTGF, VEGF, and FGF2 (growth factors) suggests that telomerase could favor cellular proliferation, reinforcing a recent study on human mammary epithelial cells (Smith et al., 2003). Also in accordance with the results from mammary epithelial cells that suggest telomerase causes resistance to the anti-proliferative effect of transforming growth factor beta, TGFBR2 was underexpressed in hTERT-BJ1 cells. Yet many other genes are differentially expressed. For example, the anti-oxidant SOD2 is downregulated by telomerase. Downregulation of SOD2 could be related to the increased resistance to apoptosis conferred by hTERT (Gorbunova et al., 2002), since SOD2 may be involved in p53-mediated apoptosis (Drane et al., 2001). IL6, an important gene in differentiation and inflammatory response is also downregulated. The expression level of several MMPs is decreased but there is also a large 3-fold decrease in tissue inhibitor of metalloproteinase 1. Lastly, IGFBP3 is overexpressed in hTERT-BJ1 HDFs, which is intriguing since IGFBP3 is overexpressed in senescent HDFs (Moerman et al., 1993). Taken as a whole, these results suggest that telomerase prevents RS and promotes proliferation but disrupts many normal cellular functions.

3.3. Characterization of gene expression in H_2O_2-induced SIPS in BJ and hTERT-BJ1 cells

In BJ cells in H_2O_2-induced SIPS, 14 genes displayed an increased- and 7 genes displayed a decreased-mRNA steady-state level (Table 2, Fig. 2A). Several are involved in cellular proliferation: e.g. increases in CTGF, EGR1, FGF2, ODC mRNA levels. The increase in EGR1 level is puzzling since EGR1 has been recently suggested, in mice, to control p53 (Krones-Herzig et al., 2003). Interleukin-1 beta (IL1B), which was previously shown to be

Table 1: Genes differentially expressed in hTERT-BJ1 HDFs when compared to normal BJ HDFs[a]

Symbol	Gene name	T/C	Function	GenBank
		Genes overexpressed as a result of the presence of telomerase		
CTGF	Connective tissue growth factor	↑10.7	Cell proliferation	U14750
CAV1	Caveolin-1	↑4.0	Signal transduction; endocytosis; potocytosis	NM_001753
IGFBP3	Insulin growth factor binding protein3	↑3.6	Signal transduction; disrupts binding of IGF-1	X64875
GADD153	DNA damage inducible transcript3	↑3.4	Stress response	S40706
CANX	Calnexin	↑3.0	Protein secretion; probably apoptosis	NM_001746
FN1	Fibronectin	↑3.0	Cell adhesion/migration	X02761
ODC	Ornithine decarboxylase1	↑2.4	Cell cycle & proliferation	NM_002539
PKM2	pyruvate-kinase-muscle 2	↑2.4	Energetic metabolism	M26252
SM22	Transgelin	↑2.2	Muscle development	M95787
MEK1	Mitogen activated protein kinase kinase1	↑2.2	Cell proliferation control	L11284
TFAP2C	Transcription factor AP2-gamma	↑2.1	Morphology	NM_003222
TPA	Plasminogen activator tissue	↑2.0	Cell migration and tissue remodeling	NM_000930
FGF2	fibroblast growth factor 2	↑2.0	Cell proliferation	NM_002006
VEGF	Vascular endothelial growth factor	↑1.8	Angiogenesis; Cell proliferation	AF022375
AOP2	Anti-oxidant-protein2	↑1.8	Defense system	NM_004905
SMAD3	SMAD3	↑1.8	Signal transduction	U68019
VEGFB	Vascular endothelial growth factor B	↑1.7	Angiogenesis; Cell proliferation	U43368
SHC	p66-SHC transforming protein1	↑1.6	Cell proliferation; apoptosis	U73377
		Genes not found expressed in BJ cells		
CATB	Catenin, beta 1	↑	Cell adhesion; transduction signal	NM_001904
		Genes underexpressed as a result of the presence of telomerase		
SOD2	Superoxide dismutase2	↓18.0	Stress response	NM_000636
IL6	Interleukin 6	↓5.6	Immune response	NM000600
APOJ	ApolipoproteinJ	↓4.1	Lipid metabolism	J02908
MMP7	Matrix metalloproteinase 7	↓4.1	Degradation of extracellular matrix	NM_002423
MYBL2	b-myb	↓4.0	Cell cycle control	X13293
CCNF	CyclinF	↓3.7	Cell cycle control	NM_001761
MMP2	Matrix metalloproteinase 2	↓3.4	Degradation of extracellular matrix	NM_004530
TIMP1	Tissue inhibitor of metalloproteinase1	↓3.2	Inhibitor of degradation of extracellular matrix	NM_003254
FES	Feline sarcoma oncogene	↓3.0	Cell proliferation	X52192
CCND3	CyclinD3	↓3.0	Cell cycle control	NM_001760
CSF1R	Colony stimulating factor 1 receptor	↓2.7	Cell proliferation	NM_005211
TFAP2B	Transcription factor AP2-beta	↓2.7	Neurogenesis; morphology	X95694
CDK6	Cyclin dependent kinase6	↓2.7	Cell proliferation	NM001259
FGF8	Fibroblast growth factor 8	↓2.3	Cell proliferation	U36223
SPRR1B	Cornifin	↓2.3	Cell structure	NM_003125
BAD	BCL2-antagonist of cell death	↓2.2	Pro-apoptotic	NM_004322
BIN1	Bridging integrator 1	↓2.2	Cell cycle control; apoptosis	NM_004305
VWF	Factor von willebrand	↓2.1	Role in blood coagulation	NM_000552
E2F2	E2F transcription factor2	↓2.1	Cell cycle control; apoptosis	NM_004091
CDH11	Cadherine11	↓1.9	calcium-dependent glycoprotein; Cell adhesion	NM_001797
CTSD	CathepsinD	↓1.9	Intracellular degradation and turnover of proteins	NM_001904
H4FM	Histone4 member M consensus	↓1.8	Cell cycle	NM_003495
IGF1R	Insulin like growth factor1 receptor	↓1.8	Cell proliferation; anti-apoptotic	NM_000875
MMP14	Matrix metalloproteinase 14	↓1.8	Degradation of extracellular matrix	NM_004995
IL11RA	Interleukin 11-receptor-alpha	↓1.8	Signal transduction	U32324
CKB	Creatin-kinase-brain	↓1.8	Energy homeostasis	M16364
MMP11	Matrix metalloproteinase 11	↓1.8	Degradation of extracellular matrix	NM_005940
TGFBR2	TGF-beta-R2	↓1.7	Cell proliferation	D50683
MMP15	Matrix metalloproteinase 15	↓1.7	Degradation of extracellular matrix	NM_002428
Ki-67	Ki-67	↓1.6	Cell proliferation	NM_002417
JUND	Jun D proto-oncogene	↓1.6	Transcription factor	NM_005354
		Genes not found expressed in hTERT-BJ1 cells		
MMP3	Matrix metalloproteinase 3	↓	Degradation of extracellular matrix	NM_002422
PAI2	Plasminogen activator inhibitor type2	↓	Fibrinolysis; Cell cycle	J02685
ESR2	Estrogen receptor beta	↓	Cell-cell signaling	X99101
BCLX	BCLX	↓	Apoptosis	NM_001191

[a]We calculated the ratio of the normalized hybridization intensity of hTERT-BJ1 cells and the BJ controls (T/C). Genes not expressed in one condition but whose value in the other condition is higher than the mean value for all genes in the corresponding array are represented as genes not found expressed.

overexpressed in senescent BJ HDFs (Shelton et al., 1999), becomes expressed in BJ cells in H_2O_2-induced SIPS.

In hTERT-BJ1 cells, 16 genes were differentially expressed in H_2O_2-induced SIPS (Table 3, Fig. 2B). Noteworthy is the increased mRNA level of $p21^{WAF-1}$ in SIPS of BJ and hTERT-BJ1 cells, which correlates with the results obtained previously at the protein level (de Magalhaes et al., 2002). Also in both cell lines, MMP3 is upregulated as a result of stress. The levels of MMP increase in aged skin, a process exacerbated by sun-exposure (Chung et al., 2001). IGFBP5 is also overexpressed in both cell lines. Since IGFBP5 disturbs the binding of IGF-1, evidence suggests it favors growth arrest and/or apoptotic pathways (Schneider et al., 2002). One previous study in BJ HDFs showed that senescent cells overexpress IGFGP5 (Shelton et al., 1999). The BCL2-associated X protein gene (BAX) is also upregulated by oxidative stress. BAX forms a heterodimer with BCL2 and functions as an apoptotic activator, mediated by p53. Its increased mRNA level suggests that some apoptotic pathways could be active as a result of a single H_2O_2 stress. MDM2 becomes expressed in BJ cells after stress, which is in contrast with BAX since MDM2 inhibits p53-mediated apoptosis. Also interesting is the downregulation of GADD153 in both cell lines since GADD153 has been previously shown to be involved in stress response (Guyton et al., 1996). In murine cells, GADD153 levels increase with age and make cells more susceptible to oxidative stress (Ikeyama et al., 2003).

There are many genes differentially expressed between the non-stressed BJ and hTERT-BJ1 cells. Therefore, comparisons between both cell lines must be taken with care. Several genes differentially expressed in SIPS of BJ cells but not in hTERT-BJ1 cells were also differentially expressed in non-stressed control hTERT-BJ1 cells when compared to non-stressed BJ cells and inversely (Tables 1, 2 and 3). For example, CTGF and CANX are overexpressed in BJ cells as a result of a single H_2O_2 stress but not in hTERT-BJ1 cells; yet these genes are already overexpressed in BJ cells compared to hTERT-BJ1 controls. Therefore, in those cases, when looking at the effects of the H_2O_2 stress on the gene profiles of BJ or hTERT-BJ1 cells, we witness a converging shift in gene expression.

3.4. Failure to induce premature senescence in WS skin fibroblasts

Based on the mortality curve obtained at day 2 after exposure of WS skin fibroblasts to H_2O_2 (Fig. 3A), we decided to use a subcytotoxic concentration of 75 μM H_2O_2. The percentage of cell survival obtained at 25, 50, and 75 μM was not different, indicating that these doses are cytotastic, given the limited 20% increase in the cell population between day 0 (stress day) and

Table 2: Genes differentially expressed in BJ HDFs in H_2O_2-induced SIPS[a]

Gene	Name	S/C	Function	GenBank	hTERT-BJ1 in SIPS
		Genes overexpressed in H_2O_2-induced SIPS			
MMP3	Matrix metalloproteinase 3	↑4.5	Degradation of extracellular matrix	NM_002422	+ expression
CTGF	Connective tissue growth factor	↑2.7	Cell proliferation	U14750	=
BAX	BCL2-associated X protein	↑2.4	Apoptosis	NM_004324	↑1.6
EGR1	Early growth response1	↑2.4	Transcriptional regulator	NM_001964	=
FGF2	fibroblast growth factor 2	↑2.3	Cell proliferation	NM_002006	=
p21	Cyclin dependent kinase inhibitor 1A	↑2.1	Cell cycle control	U03106	↑2.1
ODC	Ornithine decarboxylase1	↑1.6	Cell cycle & proliferation	NM_002539	↓1.8
CANX	Calnexin	↑1.6	Protein secretion; probably apoptosis	NM_001746	=
IGFBP5	Insulin growth factor binding protein5	↑1.6	Signal transduction; disrupts binding of IGF-1	M65062	↑2.1
		Genes not found expressed in non-stressed BJ fibroblasts			
CATB	Catenin, beta 1	↑	Cell adhesion; transduction signal	NM_001904	=
CASP9	Caspase9	↑	Apoptosis	NM_001229	=
IL1B	Interleukin1 beta	↑	Inflammatory and immune responses	M15330	=
CASP2	Caspase2	↑	Apoptosis	NM_001224	=
MDM2	MDM2	↑	Cell cycle control	NM_002392	=
		Genes underexpressed in H_2O_2-induced SIPS			
AOP2	Anti-oxidant-protein2	↓2.6	Defense system	NM_004905	=
TFAP2A	Transcription factor AP2-alpha	↓1.8	Morphology	M36711	↑1.9
VEGFR2	Vascular endothelial growth factor receptor2	↓1.7	Angiogenesis; Cell proliferation	NM_002253	=
VEGFR3	Vascular endothelial growth factor receptor3	↓1.6	Angiogenesis; Cell proliferation	NM_002020	=
GADD153	DNA damage inducible transcript3	↓1.6	Stress response	S40706	↓4.7
PAI1	Plasminogen activator inhibitor type1	↓1.6	Fibrinolysis; Cell cycle	M14083	=
IGF1R	Insulin like growth factor1 receptor	↓1.5	Cell proliferation; anti-apoptotic	NM_000875	=

[a]We calculated the ratio of the normalized hybridization intensity of BJ cells in H_2O_2-induced SIPS and the BJ controls (S/C). Genes not expressed in one condition but whose value in the other condition is higher than the mean value for all genes in the corresponding array are represented as genes not found expressed.

day 2 after the stress. 75 μM H_2O_2 is much inferior to the concentrations used to induce SIPS in AG04431, BJ, IMR-90, and WI-38 HDFs (Fig. 3B). In other words, the WS fibroblasts are much more sensitive to H_2O_2.

Contrary to other strains of fibroblasts, the percentage of SA βgal positive cells did not increase with the dose of H_2O_2 used (Fig. 3C). Also intriguing, although in accordance with previous reports (Choi et al., 2001), the percentage of WS fibroblasts positive for SA βgal in the control was relatively low (10.4%) when compared to pre-senescent WI-38, IMR-90, or BJ fibroblasts.

We used the low-density DNA arrays described above to compare gene expression patterns between WS fibroblasts and both young and old BJ cells. Although AG00780 and BJ cell lines are derived from the skin, it is difficult to determine if alterations in gene expression are due to the WRN protein or to differences between the cell lines. Even so, we found a higher percentage of genes altered when comparing WS fibroblast and young BJ cells than when comparing old BJ cells and WS fibroblasts (20 versus 12%), though this pattern concerns a high diversity of genes.

The list of genes that are differentially expressed in WS fibroblasts when compared to young BJ cells and not differentially expressed when compared to old BJ cells is given in Table 4. The number of these changes was quite limited and concerned 26 genes. The most striking changes are observed for IL11RA, which is involved in resistance against apoptosis (Leng et al., 1997) and is down-regulated by 64%. MSRA, involved in protection of proteins against oxidative stress and GSTPi, a glutathione transferase also involved in protection against oxidative stress, roughly decreased by half. FGF receptor is decreased by 40%. Regarding genes overexpressed, we found an overexpression of $p66^{shc}$, a downstream target of p53 involved in apoptosis (Trinei et al., 2002). These results explain in part the increased sensitivity of WS cells to oxidative stress. SM22 has been found to be overexpressed in senescent IMR-90 and WI-38 HDFs and is overexpressed in WS cells. Pyruvate kinase protein level was found to increase in senescent WI-38 HDFs (Dierick et al., 2002) and the mRNA level of this gene shows a 3.3-fold increase in WS cells. Caveolin-1 is phosphorylated in NIH-3T3 cells in H_2O_2-induced SIPS and its overexpression causes premature senescence (Volonte et al., 2002), and a 3.7-fold increase was found in WS cells. PAI2 overexpression is a marker of senescence in normal HDFs and a 4.6-fold increase was found in WS cells. Lastly, PCNA was overexpressed, as it was in SIPS in hTERT-BJ1 cells. PCNA is involved in multiple functions, such as DNA repair, and some evidence suggests it is regulated by p53 through $p21^{WAF-1}$ (Xu and Morris, 1999). The

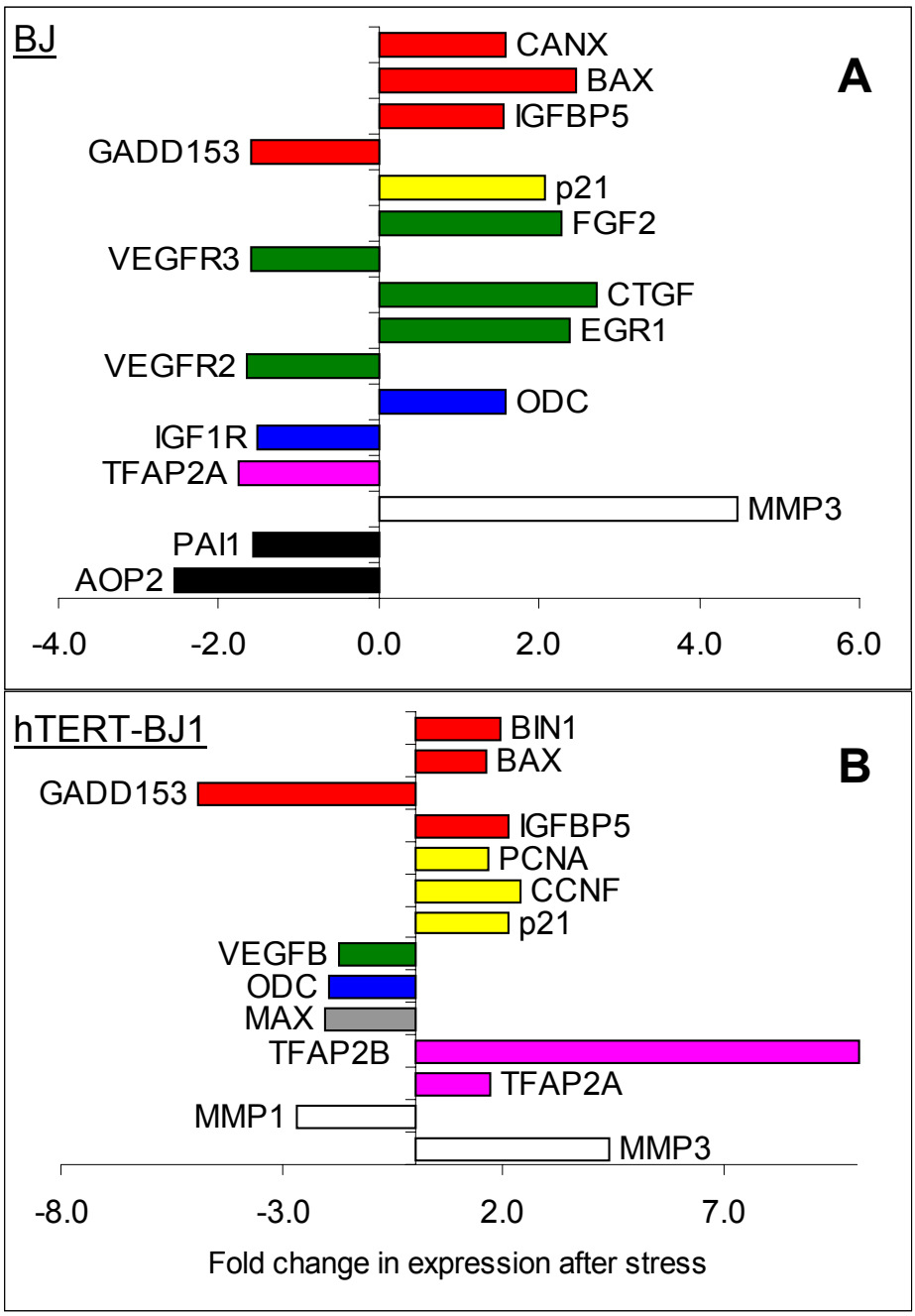


Figure 2: Genes differentially expressed in BJ (A) and hTERT-BJ1 (B) fibroblasts in H_2O_2-induced SIPS. Values represent the ratio of the normalized hybridization intensity. Each gene has been classified by function according to what best suits its role based on the present literature: Pro-apoptotic = Red; Anti-proliferative = Yellow; Pro-proliferative = Green; Anti-apoptotic = Blue; Morphology = Pink; Pro- or anti-apoptotic depending on context = Gray; Degradation of extracellular matrix = White; Miscellaneous = Black.

overexpression of PCNA appears to be part of the cellular response to DNA damage both in hTERT-BJ1 cells in SIPS and in WS fibroblasts.

3.5. Gene expression in senescent BJ cells versus BJ cells at early PD

We also compared young versus old BJ cells (Table 5). As previously mentioned, gene expression changed more between young and old BJ cells than when we submitted young BJ cells to H_2O_2 stress. Although some key genes were common between SIPS and RS (e.g. $p21^{WAF-1}$), the degree of gene expression shift between SIPS and RS suggests that SIPS and RS are not exactly the same process. It remains to be seen which of the processes, RS or SIPS, is predominant in the in vivo degeneration of tissues, namely stress-prone tissues like the skin. MMP3 and MMP1 are upregulated in old BJ cells, as happens in aged or photoaged skin (Chung et al., 2001), perhaps as a result of $p21^{WAF-1}$ activity (Perlman et al., 2003). IGFBP5, PAI2, IL-6, caveolin-1, pyruvate kinase, etc. are also upregulated by senescence, as reported by others (Shelton et al., 1999; Dierick et al., 2002; Volonte et al., 2002; Zhang et al., 2003). CCNF and MYBL2 are downregulated, as has been previously reported (Shelton et al., 1999; Ly et al., 2000). A decrease of 2.1-fold of IGF1-receptor is also worth to be reported, given its potential role in resistance against apoptosis.

3.6. DNA-binding activity of p53, HIF, CREB, AP-1, NF-κB, PPARg, and NFAT

Although DNA arrays offer a picture of the changes that cells undergo during SIPS, they cannot by themselves offer an explanation as to what mechanisms regulate the process. Therefore, we studied the activity of several transcription factors at different times after a single H_2O_2 stress in an attempt to uncover the underlying mechanisms responsible for SIPS.

The DNA-binding activity of p53 increases after a single H_2O_2 stress in both BJ (Fig. 4A) and hTERT-BJ1 cells (Fig. 4B). There appears to be a peak of p53's DNA-binding activity at 2 hrs after the stress, which stabilizes at 6 hrs. Interestingly, 72 hrs after the stress, p53 activity is still considerably higher (+/- 2-fold) than in control cells. In contrast, p53 protein level upregulation has been previously shown in BJ and hTERT-BJ cells at 24 hrs after stress (Morales et al., 1999), but not at 72 hrs (de Magalhaes et al., 2002). In addition, results from IMR-90 fibroblasts showed that p53 upregulation after stress fades in a few days, which led to the conclusion that the role of p53 in SIPS was minor (Chen et al., 1998, 2000). Finally, the basal level of p53 DNA-binding activity is slightly lower in hTERT-BJ1 cells when compared to BJ controls, suggesting that this difference may be responsible for the SOD2 downregulation in hTERT-BJ1 cells (Drane et al., 2001).

Table 3: Genes differentially expressed in hTERT-BJ1 HDFs in H_2O_2-induced SIPS[a]

Symbol	Gene name	S/C	Function	GenBank	BJ in SIPS
		Genes overexpressed in H_2O_2-induced SIPS			
TFAP2B	Transcription factor AP2-beta	↑13.6	Neurogenesis; morphology	X95694	=
CCNF	CyclinF	↑3.1	Cell cycle control	NM_001761	=
p21	Cyclin dependent kinase inhibitor 1A	↑2.1	Cell cycle control	U03106	↑2.1
IGFBP5	Insulin growth factor binding protein5	↑2.1	Signal transduction; disrupts binding of IGF-1	M65062	↑1.6
TFAP2A	Transcription factor AP2-alpha	↑1.9	Morphology	M36711	↓1.8
BIN1	Bridging integrator 1	↑1.8	Cell cycle control; apoptosis	NM_004305	=
PCNA	Proliferating cell nuclear antigen	↑1.6	Control of DNA replication	NM002592	=
BAX	BCL2-associated X protein	↑1.6	Apoptosis	NM_004324	↑2.4
		Genes not found expressed in non-stressed hTERT-BJ1 fibroblasts			
MMP3	Matrix metalloproteinase 3	↑	Degradation of extracellular matrix	NM_002422	↑4.5
		Genes underexpressed in H_2O_2-induced SIPS			
GADD153	DNA damage inducible transcript3	↓4.7	Stress response	S40706	↓1.6
MMP1	Matrix metalloproteinase 1	↓2.8	Degradation of extracellular matrix	NM_002421	=
MAX	MAX protein	↓2.2	Cell proliferation control	NM_002382	=
ODC	Ornithine decarboxylase1	↓1.8	Cell cycle & proliferation	NM_002539	↑1.6
VEGFB	Vascular endothelial growth factor B	↓1.6	Angiogenesis; Cell proliferation	U43368	=
		Genes not found expressed in hTERT-BJ1 cells in H_2O_2-induced SIPS			
HMOX	Heme-oxygenase	↓	Defense system	NM_002133	=
KNSL6	Mitotic-centromere-associated-kinesin	↓	Cell proliferation	NM_006845	=

[a]We calculated the ratio of the normalized hybridization intensity of hTERT-BJ1 cells in H_2O_2-induced SIPS and the respective controls (S/C). Genes not expressed in one condition but whose value in the other condition is higher than the mean value for all genes in the corresponding array are represented as genes not found expressed.

It is clear that HIF-1's DNA-binding activity increases as a result of a single H_2O_2 stress, both in BJ (Fig. 4C) and hTERT-BJ1 (Fig. 4D) cells. This data is in agreement with the mitochondrial model of HIF-1 in which oxidative stress activates HIF-1, meaning that H_2O_2 might directly activate HIF-1 (Michielis et al., 2002). The increase in DNA-binding activity of HIF-1 can help explain the overexpression of FGF2 in H_2O_2-induced SIPS since FGF2 levels have been suggested to be mediated by HIF-1 (Li et al., 2002).

Although the DNA-binding activity of NF-κB does not appear to be altered during the 72 hrs after a single H_2O_2 stress, we noticed that the basal NF-κB DNA-binding activity is at least 2-fold higher in BJ cells compared to hTERT-BJ1 (243 ± 82%). On the contrary, phosphorylated ATF-2's basal DNA-binding activity is much higher in hTERT-BJ1 cells compared to BJ controls (at least 3-fold since the DNA-binding activity increases 371 ± 60%). Regarding the other studied transcription factors (AP-1, CREB, NFAT, PPARg), we did not find any significant differences in DNA-binding activity either as a result of a single H_2O_2 stress or between BJ and hTERT-BJ1 cells (data not shown).

4. Discussion

It was reported that $p21^{WAF-1}$ is overexpressed at mRNA and protein levels in H_2O_2-induced SIPS in IMR-90 (Chen et al., 1998, 2000) and BJ HDFs (de Magalhaes et al., 2002). $p21^{WAF-1}$ inhibits the phosphorylation of the retinoblastoma protein by cyclin-dependent kinases, explaining the growth arrest observed in SIPS and the appearance of biomarkers of senescence such as SA β-gal, senescent morphology, etc. This overexpression of $p21^{WAF-1}$ can be due to an increased DNA-binding activity of p53, as observed here from 2 to 72 hrs after stress. Upregulation of p53's protein level had been reported previously at 24 (Morales et al. 1999) but not at 72 hrs after stress in BJ cells (de Magalhaes et al., 2002). Our DNA array results also did not show any significant change in the steady-state mRNA level of p53 following H_2O_2 stress in either cell line. Yet evidence suggests that post-translational modifications activate p53 in high-passage HDFs (Atadja et al., 1995), explaining why p53's DNA-binding activity, but not protein or mRNA levels, increases at 72 hrs after H_2O_2 stress. Although p53-independent induction of $p21^{WAF-1}$ has been reported, $p21^{WAF-1}$ activation due to DNA damage probably involves p53 (Michieli et al., 1994). Nevertheless, a p53-independent induction of $p21^{WAF-1}$ is possible since IMR-90 HDFs lacking p53 due to ectopic expression of the viral protein E6 are still able to develop a senescent phenotype after subcytotoxic H_2O_2 stress, while cells lacking E7 cannot, since pRb is necessary for SIPS to occur (Chen et al., 2000). Moreover, the kinase activity of

Table 4: Genes differentially expressed in WS fibroblasts when compared to young BJ HDFs and not differentially expressed when compared to old BJ HDFs[a]

Symbol	Gene name	WS/Y	Function	GenBank	Old vs. Young
		Genes overexpressed in WS fibroblasts			
PAI2	Plasminogen activator inhibitor type2	↑4.6	Fibrinolysis; Cell cycle	J02685	↑3.2
ANX1	Annexin1	↑3.7	Anti-inflammatory	NM_000700	↑3.1
CAV1	Caveolin-1	↑3.7	Signal transduction; endocytosis; potocytosis	NM_001753	↑2.9
PKM2	pyruvate-kinase-muscle 2	↑3.3	Energetic metabolism	M26252	↑3.1
PCNA	Proliferating cell nuclear antigen	↑2.6	Control of DNA replication	NM002592	↑2.5
CANX	Calnexin	↑2.6	Protein secretion; probably apoptosis	NM_001746	=
SM22	Transgelin	↑2.4	Muscle development	M95787	=
CTSB	CathepsinB	↑2.3	Intracellular degradation and turnover of proteins	NM_001904	=
TB10	Thymosin beta 10	↑2.3	Cell motility	NM_021103	=
CCND1	CyclinD1	↑2.2	Cell cycle control	NM_053056	=
RRM1	Ribonucleotide-reductase M1	↑2.1	DNA synthesis	NM_001033	=
ITGB1	Integrin beta1	↑2	Cell adhesion	NM_002211	=
VEGFC	Vascular endothelial growth factor C	↑1.9	Angiogenesis; Cell proliferation	NM_005429	=
HSP70	Heat shock 70kD protein1	↑1.8	Defense system	AB023420	=
SHC	p66-SHC transforming protein1	↑1.7	Cell proliferation; apoptosis	U73377	=
		Genes underexpressed in WS fibroblasts			
FHIT	Fragile histidine triad gene	↓2.9	Purine metabolism	NM_002012	=
IL11RA	Interleukin 11-receptor-alpha	↓2.8	Signal transduction	U32324	=
Ki-67	Ki-67	↓2.8	Cell proliferation	NM_002417	=
MMP11	Matrix metalloproteinase 11	↓2.3	Degradation of extracellular matrix	NM_005940	=
MSRA	Methionine-sulfoxide-reductase A/peptide	↓2.3	Stress response	AF183420	=
CSF1R	Colony stimulating factor 1 receptor	↓2.2	Cell proliferation	NM_005211	=
GSTPi	Glutathione S-transferase pi	↓1.8	Detoxification	NM_000852	=
MMP15	Matrix metalloproteinase 15	↓1.7	Degradation of extracellular matrix	NM_002428	=
FGFR	Fibroblast growth factor receptor 1	↓1.7	Cell proliferation	NM_000604	=
ICAM-1	Intracellular adhesion molecule1	↓1.6	Cell adhesion	J03132	=
NCOR1	Nuclear receptor co-repressor 1	↓1.6	Transcription	NM_006311	=

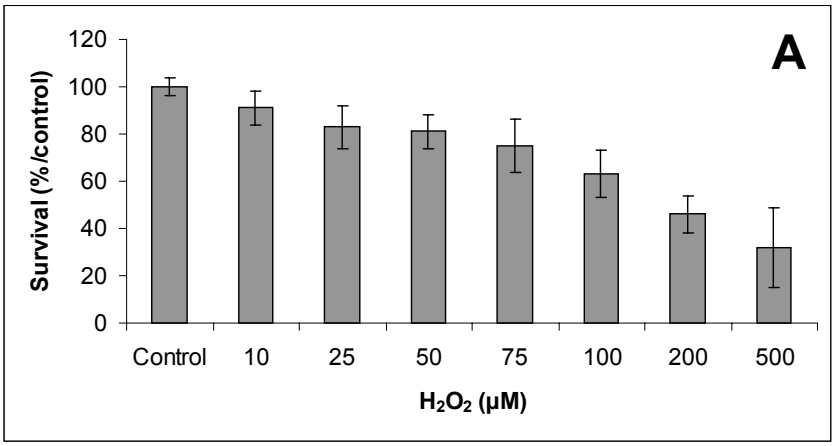
[a]We calculated the ratio of the normalized hybridization intensity of WS fibroblasts and young BJ cells (WS/Y). Only genes not differentially expressed between WS fibroblasts and old BJ HDFs were considered.

cyclin-dependent kinase 2 has been shown to be greatly decreased in a $p21^{WAF-1}$-independent manner in IMR-90 cells (Frippiat et al., 2003), suggesting other players may be involved.

In accordance with our previous results (de Magalhaes et al., 2002), the expression of telomerase does not appear to protect or interfere with the appearance of SIPS or its general mechanisms occurring through $p21^{WAF-1}$ overexpression. One plausible explanation is that disruption of the telomeres, rather than telomere shortening, induces SIPS. Indeed, exposure to oligonucleotides homologous to the telomere 3'-overhang sequence induces a senescent phenotype (Li et al., 2003). Yet telomerase appears to stabilize the telomeres (Blackburn, 2000). The inability of telomerase to interfere with SIPS suggests generalized DNA damage generated by H_2O_2 as another possible mechanism in SIPS.

The two main features of HDFs in SIPS are that cells remain alive despite exposure to stress (they do not apoptose) and their cycle is irreversibly blocked. In normal conditions a balance exists between the pro- and anti-proliferative signals and the pro- and anti-apoptotic signals. It could be that, in SIPS, this particular multi-variable balance gets reorganized, still allowing cell survival but blocking the cell cycle. The activation of pro-proliferative and anti-apoptotic signal transduction pathways could inhibit pro-apoptotic pathways while the activation of anti-proliferative pathways inhibit growth, e.g. $p21^{WAF-1}$ overexpression. For instance, p53 is known to regulate the expression level of $p21^{WAF-1}$ while the pro-apoptotic gene BAX is also known to be regulated by p53 (Schuler et al., 2003). On the other hand, p53 may regulate HIF-1, involved in the adaptation against oxidative stress. Indeed, activation of p53 by oxidative stress can result in either apoptosis or growth arrest and the events that determine the decision remain unclear (Martindale and Holbrook, 2002). Other examples exist, such as the overexpression of IGFBP5 or the downregulation of IGF1R. In addition, we witness the downregulation of the pro-apoptotic GADD153. GADD153 has been shown to be involved in the stress response to UV damage and oxidative stress (Guyton et al., 1996). In Hela cells, GADD153 is overexpressed after oxidative stress, not underexpressed as occurs in our model. The expression of the GADD153 pro-apoptotic gene in hepatocytes increases with age in mice and makes cells more susceptible to oxidative stress (Ikeyama et al., 2003). It is interesting to notice how both anti- and pro-apoptotic genes can be up- and downregulated as if to keep the balance between them (Fig. 2).

Since previous reports indicated that hTERT transfection into BJ cells did not alter normal cellular functions, for instance hTERT did not alter the karyotype (Bodnar et al., 1998; Jiang et al., 1999; Morales et al., 1999), it was surprising to find so many differentially expressed genes between BJ and hTERT-BJ1 cells. Such abrupt differences indicate that the presence of



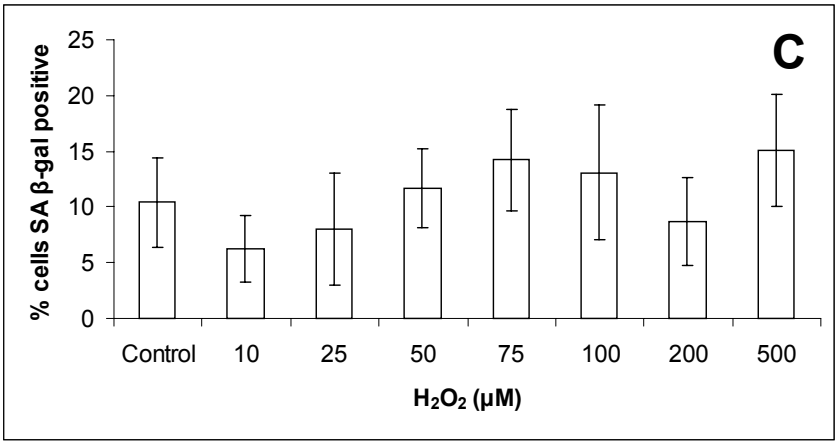


Figure 3: A. Cytotoxicity of a single exposure to H_2O_2 of AG00780 HDFs. H_2O_2 doses ranged from 10 to 500 µM. The mortality was estimated by measuring LDH release 48 hrs after the stress. The results are expressed as percentage of the values found in control cells submitted to the same culture conditions than the stressed cells but without any H_2O_2. Results are given as mean values ± S.D. from three independent experiments.

B. Comparison of the subcytotoxic concentration of H_2O_2 (in µM) used during a 2-h stress to induce SIPS in different lines of HDFs.

C. Effects of a single H_2O_2 stress exposure on the proportion of AG00780 HDFs positive for SA βgal activity. H_2O_2 concentrations ranged from 10 to 500 µM. Controls represent the HDFs submitted to the same culture conditions than the stressed cells but without any H_2O_2. Results represent the proportion of cells positive for SA βgal. The results are presented as mean values ± S.D. from three independent experiments.

Comparison of subcytotoxic concentration of H_2O_2 inducing SIPS in different lines of HDFs

B

HDFs	Concentration of H_2O_2 (µM)
AG00780	75
IMR-90	150
AG04431	300
WI-38	300
BJ	1200
hTERT-BJ1	1200

telomerase does more than avoid the end-replication problem, as suggested by other recent results (Smith et al., 2003). This suggests that telomerase-immortalized cells, while not being transformed, do not have the same functional abilities of normal cells. In addition, recent results hint SOD2 as a tumor suppressor (Plymate et al., 2003). The observed downregulation of SOD2 in hTERT-BJ1 HDFs confirms other reports that telomerase may favor tumorigenesis by a telomere length-independent mechanism (Stewart et al., 2002; Lindvall et al., 2003).

Our data confirms previous reports that WS fibroblasts are more sensitive to oxidative stress than normal cells. Based on our results, WS fibroblasts do not develop SIPS. Previous reports indicated that SA βgal in AG00780 fibroblasts increases dramatically from PD 10.9 to PD 32.5 (Choi et al., 2001). Therefore, according to this biomarker the WS fibroblasts are not senescent at early PDs and did not get senescent when submitted to oxidative stress, which suggests a complex regulation of SA βgal in WS cells.

An old question is whether the effects of mutations in the WRN protein mimic the process of normal senescence. Our gene expression results suggest that similar pathways might be at work. The mutations in the WRN protein are suggested to induce changes that become irreversible. In agreement with this idea, ectopic expression of telomerase can immortalize WS fibroblasts, and nevertheless gene expression patterns remain abnormal (Choi et al., 2001). More globally, our results suggest that telomerase, although it increases the proliferative capacity of cells, alters the functionality of cells and, as happens when expressing telomerase ectopically in WS and progeria cells (Bai and Murnane, 2003), does not fully restore the cellular functions to young cells.

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Table 5: Genes differentially expressed in old, pre-senescent BJ HDFs when compared to BJ HDFs at early PDs[a]

Symbol	Gene name	O/Y	Function	GenBank
		Genes overexpressed in old BJ fibroblasts		
MMP3	Matrix metalloproteinase 3	↑35.4	Degradation of extracellular matrix	NM_002422
MMP1	Matrix metalloproteinase 1	↑6.5	Degradation of extracellular matrix	NM_002421
IL6	Interleukin 6	↑4.5	Immune response	NM000600
IGFBP5	Insulin growth factor binding protein5	↑4.4	Signal transduction	M65062
PLAU	Urokinase	↑3.6	Regulation of cell-surface plasminogen activation	NM_002658
PAI2	Plasminogen activator inhibitor type2	↑3.2	Fibrinolysis; Cell cycle	J02685
PKM2	pyruvate-kinase-muscle 2	↑3.1	Energetic metabolism	M26252
ANX1	Annexin1	↑3.1	Anti-inflammatory	NM_000700
CTGF	Connective tissue growth factor	↑3.0	Cell proliferation	U14750
CAV1	Caveolin-1	↑2.9	Signal transduction; endocytosis; potocytosis	NM_001753
S100A	S100 calcium binding protein A4	↑2.7	Cell cycle control	NM_002961
p21	Cyclin dependent kinase inhibitor 1A	↑2.5	Cell cycle control	U03106
PCNA	Proliferating cell nuclear antigen	↑2.5	Control of DNA replication	NM002592
FGF2	fibroblast growth factor 2	↑2.3	Cell proliferation	NM_002006
CDC42	Cell division cycle42	↑2.2	Cell cycle	NM_001791
AOP2	Anti-oxidant-protein2	↑1.7	Defense system	NM_004905
		Genes not found expressed in young BJ fibroblasts		
EGFR	Epidermal growth factor receptor	↑	Control of cell growth and differentiation	NM_005228
CATB	Catenin, beta 1	↑	Cell adhesion; transduction signal	NM_001904
SMAD2	SMAD2	↑	Signal transduction	U68018
IL1B	Interleukin1 beta	↑	Inflammatory and immune responses	M15330
		Genes underexpressed in old BJ fibroblasts		
FES	Feline sarcoma oncogene	↓2.9	Cell proliferation	X52192
HMOX	Heme-oxygenase	↓2.6	Defense system	NM_002133
CCNF	CyclinF	↓2.4	Cell cycle control	NM_001761
APOJ	ApolipoproteinJ	↓2.4	Lipid metabolism	J02908
SPRR1B	Cornifin	↓2.2	Cell structure	NM_003125
MYBL2	b-myb	↓2.1	Cell cycle control	X13293
IGF1R	Insulin like growth factor1 receptor	↓2.1	Cell proliferation; anti-apoptotic	NM_000875
E2F2	E2F transcription factor2	↓2.1	Cell cycle control; apoptosis	NM_004091
CSF1R	Colony stimulating factor 1 receptor	↓2	Cell proliferation	NM_005211
BIN1	Bridging integrator 1	↓2	Cell cycle control; apoptosis	NM_004305
FGF8	Fibroblast growth factor 8	↓1.9	Cell proliferation	U36223
BAD	BCL2-antagonist of cell death	↓1.9	Pro-apoptotic	NM_004322
CASP3	Caspase3	↓1.9	Apoptosis	NM_004346
		Gene not found expressed in old BJ fibroblasts		
ESR2	Estrogen receptor beta	↓	Cell-cell signaling	X99101

[a]We calculated the ratio of the normalized hybridization intensity of old and young BJ cells (O/Y). Genes not expressed in one condition but whose value in the other condition is higher than the mean value for all genes in the corresponding array are represented as genes not found expressed.

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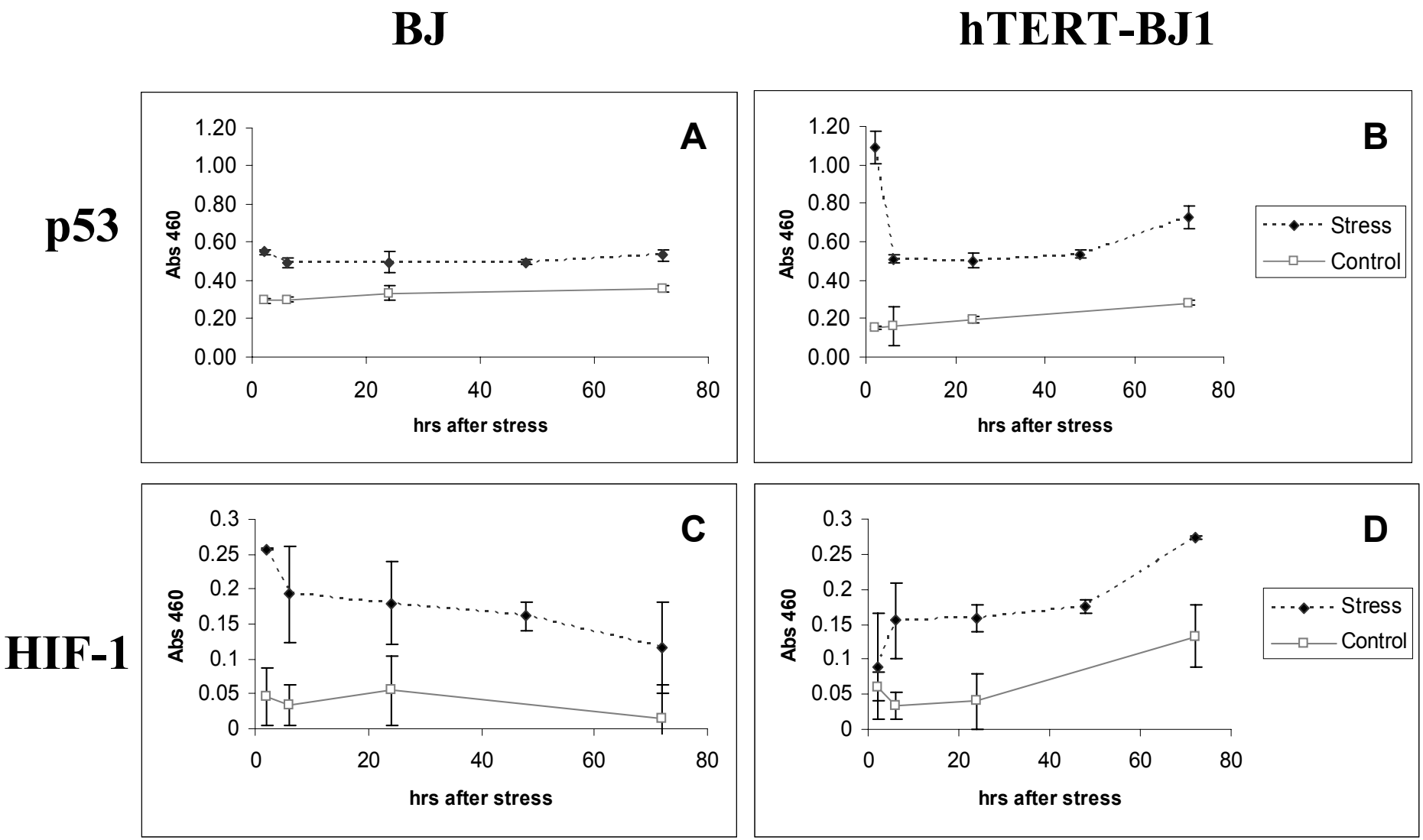

Figure 4: Effect of a single H_2O_2 stress on the p53 (A, B) and HIF-1 (C, D) DNA-binding activity in BJ (A, C) and hTERT-BJ (B, D) cells. Samples were taken at 2, 6, 24, 48, and 72 hrs after stress.

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Supplemental Data: List of the 202 genes represented on the DualChip Human General array

Symbol	Gene	General Function	Gene bank
23kd	23KDa Highly basic protein	Protein synthesis	X56932
ACTB	Beta-Actin	Cell motility	NM_001101
ADAM1	A disintegrin and metalloproteinase	Cell migration	XM_090479
ADPRT	Polysynthetase	Cell proliferation	J03473
Aldo	Aldolase A,	Glycolysis	NM_000034
ANX1	Annexin1	Anti-inflammatory	NM_000700
AOP2	Anti-oxidant-protein2	Defense system	NM_004905
APOJ	ApolipoproteinJ	Lipid metabolism	J02908
ATM	Ataxia telangiectasia mutated	Protein kinase; Cell cycle control	U26455
BAD	BCL2-antagonist of cell death	Pro-apoptotic	NM_004322
BAX	BCL2-associated X protein	Apoptosis	NM_004324
BCL2	B-cell lymphoma2	Inhibitor of Apoptosis	NM_000633
BCLX	BCLX	Apoptosis	NM_001191
BID	BH3 interacting domain death agonist	Apoptosis	NM_001196
BIN1	Bridging integrator 1	Cell cycle control; apoptosis	NM_004305
BMP2	Bone morphogenetic protein2	Cell-cell signaling	NM_001200
BSG	Basigin	Immonoglobulin superfamily	NM_001728
CANX	Calnexin	Protein secretion; probably apoptosis	NM_001746
CASP2	Caspase2	Apoptosis	NM_001224
CASP3	Caspase3	Apoptosis	NM_004346
CASP7	Caspase7	Apoptosis	NM_001227
CASP8	Caspase8	Apoptosis	X98172
CASP9	Caspase9	Apoptosis	NM_001229
CATB	Catenin, beta 1	Cell adhesion; transduction signal	NM_001904
CAV1	Caveolin-1	Signal transduction; endocytosis; potocytosis	NM_001753
CCNA1	CyclinA1	Cell cycle control	NM_003914
CCNB1	CyclinB1	Cell cycle control	NM_031966
CCND1	CyclinD1	Cell cycle control	NM_053056
CCND2	CyclinD2	Cell cycle control	NM_001759
CCND3	CyclinD3	Cell cycle control	NM_001760
CCNE1	CyclinE	Cell cycle control	NM_001238
CCNF	CyclinF	Cell cycle control	NM_001761
CCNH	CyclinH	Cell cycle control	NM_001239
CDC42	Cell division cycle42	Cell cycle	NM_001791
CDH1	Cadherine1/E-cadherine	calcium-dependent glycoprotein; Cell adhesion	NM004360
CDH11	Cadherine11	calcium-dependent glycoprotein; Cell adhesion	NM_001797
CDH13	Cadherine13	calcium-dependent glycoprotein; Cell adhesion	U59289
CDK2	Cyclin dependent kinase2	Cell proliferation	NM_001798
CDK4	Cyclin dependent kinase4	Cell proliferation	U79269
CDK6	Cyclin dependent kinase6	Cell proliferation	NM001259
CENPF	Mitosin	DNA replication	U30872
CKB	Creatin-kinase-brain	Energy homeostasis	M16364
cmyc	c-myc	Oncogenesis	NM012333
COL6A2	CollagenVI-alpha2	Extracellular matrix	NM_001849
COX2	Prostaglandin endoperoxidase synthase 2	Cell migration/angiogenesis	NM_000963
CROC1A	Ubiquitin (E2 variant1)	Cell cycle control	NM_003349
CSF1	Colony stimulating factor 1	Cell proliferation	M37435
CSF1R	Colony stimulating factor 1 receptor	Cell proliferation	NM_005211
CTGF	Connective tissue growth factor	Cell proliferation	U14750
CTSB	CathepsinB	Intracellular degradation and turnover of proteins	NM_001904
CTSD	CathepsinD	Intracellular degradation and turnover of proteins	NM_001904

Symbol	Gene	General Function	Gene bank
CTSL	CathepsinL	Intracellular degradation and turnover of proteins	NM_001912
cyc	Cyclophilin 33A	Synthesis proteins	AF042385
DHFR	Dihydrofolate reductase	Cell cycle	NM_000791
DP1	Transcription factor Dp-1	Cell cycle control	NM_007111
DP2	Transcription factor Dp-2	Cell cycle control	NM_006286
E2F2	E2F transcription factor2	Cell cycle control; apoptosis	NM_004091
E2F3	E2F transcription factor3	Cell cycle control	NM_001949
E2F4	E2F transcription factor4	Cell cycle control	NM_001950
EGFR	Epidermal growth factor receptor	Control of cell growth and differentiation	NM_005228
EGR1	Early growth response1	Transcriptional regulator	NM_001964
EGR3	Early growth response3	Transcription factor	NM_004430
EIF4	Eukaryotic translation initiation	Protein synthesis	NM_001968
EMS1	EMS1	Regulation of cell adhesion	NM_005231
ESR2	Estrogen receptor beta	Cell-cell signaling	X99101
ETFB	Electron-transfert-flavoprotein-beta	Energy generation	NM_001985
FES	Feline sarcoma oncogene	Oncogenesis	X52192
FGF2	fibroblast growth factor 2	Cell proliferation	NM_002006
FGF8	Fibroblast growth factor 8	Cell proliferation	U36223
FGFR	Fibroblast growth factor receptor 1	Cell proliferation	NM_000604
FHIT	Fragile histidine triad gene	Purine metabolism	NM_002012
FN1	Fibronectin	Cell adhesion/migration	X02761
FOS	c-fos	Oncogenesis	NM_005252
G6PD	Glucose-6-phosphate-dehydrogenase	Energetic metabolism	NM_000402
GADD153	DNA damage inducible transcript3	Cell stress	S40706
GAPD	Glyceraldehyde-3-phosphate-dehydrogenase	Glycolysis	NM002046
GLB1	Beta1-galactosidase	Glycoprotein degradation	M34423
GPX	Glutathione peroxidase	Defense system	M21304
GRB2	Growth factor receptor-bound protein2	Cell proliferation control	NM_002086
GSN	Gelsoline	Cell mobility	X04412
GSTPi	Glutathione S-transferase pi	Detoxification	NM_000852
H2B/S	Histone2b member B/S consensus	Cell cycle	NM_080593
H3FF	Histone3 member F consensus	Cell cycle	NM_003533
H4FM	Histone4 member M consensus	Cell cycle	NM_003495
HK1	Hexokinase	Glycolytic enzyme	M75126
HMOX	Heme-oxygenase	Defense system	NM_002133
HPRT	Hypoxanthine phosphoribosyltransferase 1	HouseKeeping gene	NM_000194
HSP27	Heat shock 27kD protein1	Defense system	AB020027
HSP40	Heat shock 40kD protein1	Defense system	D49547
HSP70	Heat shock 70kD protein1	Defense system	AB023420
HSP90-a	Heat shock 90kD protein1 alpha	Defense system	X15183
HSP90-b	Heat shock 90kD protein 1, beta	Defense system	NM_007355
ICAM-1	Intracellular adhesion molecule1	Cell adhesion	J03132
IGF1	Insulin like growth factor1	Cell proliferation control	X57025
IGF1R	Insulin like growth factor1 receptor	Cell proliferation; anti-apoptotic	NM_000875
IGFBP2	Insulin growth factor binding protein2	Signal transduction; disrupts binding of IGF-1	M35410
IGFBP3	Insulin growth factor binding protein3	Signal transduction; disrupts binding of IGF-1	X64875
IGFBP5	Insulin growth factor binding protein5	Signal transduction; disrupts binding of IGF-1	M65062
IL10	Interleukin 10	Immune response	NM_000572
IL11	Interleukin 11	Inflammatory responses	NM_000641
IL11RA	Interleukin 11-receptor-alpha	Signal transduction	U32324
IL15	Interleukin 15	Immune response	NM_000585
IL1A	Interleukin1 alpha	Inflammatory responses	NM000575
IL1B	Interleukin1 beta	Inflammatory and immune responses	M15330
IL4	Interleukin 4	Immune response	NM_000589

Symbol	Gene	General Function	Gene bank
IL6	Interleukin 6	Immune response	NM000600
IL8	Interleukin 8	Cell adhesion/migration	NM_000584
ING1	Inhibitor of growth family, member 1	Cell proliferation	NM005537
ITGA5	Integrin alpha5	Cell adhesion	NM_002205
ITGA6	Integrin alpha6	Cell adhesion	NM_000210
ITGB1	Integrin beta1	Cell adhesion	NM_002211
JNK1	Mitogen activated protein kinase8	Stress response	L26318
JNK2	Mitogen activated protein kinase9	Stress response	U09759
JNK3	mitogen-activated protein kinase 10	Stress response	NM_002753
JUND	Jun D proto-oncogene	Transcription factor	NM_005354
Ki-67	Ki-67	Cell proliferation	NM_002417
KNSL5	Mitotic-kinesin-like-protein1	Cell proliferation	NM_004856
KNSL6	Mitotic-centromere-associated-kinesin	Cell proliferation	NM_006845
MAX	MAX protein	Cell proliferation control	NM_002382
MDH	Malate dehydrogenase 1	Citric acid cycle	NM_005917
MDM2	MDM2	Cell cycle control	NM_002392
MEK1	Mitogen activated protein kinase kinase1	Cell proliferation control	L11284
MEK2	Mitogen activated protein kinase kinase2	Cell mobility	NM_030662
MMP1	Matrix metalloproteinase 1	Degradation of extracellular matrix	NM_002421
MMP11	Matrix metalloproteinase 11	Degradation of extracellular matrix	NM_005940
MMP12	Matrix metalloproteinase 12	Degradation of extracellular matrix	NM_002426
MMP13	Matrix metalloproteinase 13	Degradation of extracellular matrix	NM_002427
MMP14	Matrix metalloproteinase 14	Degradation of extracellular matrix	NM_004995
MMP15	Matrix metalloproteinase 15	Degradation of extracellular matrix	NM_002428
MMP2	Matrix metalloproteinase 2	Degradation of extracellular matrix	NM_004530
MMP3	Matrix metalloproteinase 3	Degradation of extracellular matrix	NM_002422
MMP7	Matrix metalloproteinase 7	Degradation of extracellular matrix	NM_002423
MMP9	Matrix metalloproteinase 9	Degradation of extracellular matrix	NM_004994
MSRA	Methionine-sulfoxide-reductase A/peptide	Stress response	AF183420
MYBL2	b-myb	Cell cycle control	X13293
NCK1	NCK adaptor protein1	Cell cycle	NM_006153
NCOR1	Nuclear receptor co-repressor 1	Transcription	NM_006311
NCOR2	Nuclear receptor co-repressor 2	Cell cycle	NM_006312
ODC	Ornithine decarboxylase1	Cell cycle & proliferation	NM_002539
ON	Osteonectin	Ossification	NM_003118
OPN	Osteopontin	Ossification	NM_000582
p16	Cyclin dependent kinase inhibitor 2A	Cell cycle control	L27211
p21	Cyclin dependent kinase inhibitor 1A	Cell cycle control	U03106
p27	Cyclin dependent kinase inhibitor 1B	Cell cycle control	NM_004064
p35	Cyclin dependent kinase5 regulatory subunit1	Cell proliferation	NM_003885
p53	Tumor protein p53	regulation of cell cycle	AF307851
p57	Cyclin dependent kinase inhibitor 1C	regulation of cell cycle	NM_000076
PAI1	Plasminogen activator inhibitor type1	Fibrinolysis; Cell cycle	M14083
PAI2	Plasminogen activator inhibitor type2	Fibrinolysis; Cell cycle	J02685
PAK	P21 activated kinase1	Cell mobility/morphology	NM_002576
PCNA	Proliferating cell nuclear antigen	Control of DNA replication	NM002592
PGR	Progesterone receptor	Cell-cell signaling	NM_000926
PKM2	pyruvate-kinase-muscle 2	Energetic metabolism	M26252
PLA2	Phospholipase A2	Signal transduction	M86400
PLAU	Urokinase	Regulation of cell-surface plasminogen activation	NM_002658
PLK	Polo-like kinase	Cell cycle control	U01038
POLA2	Polymerase alpha	Cell cycle	NM_002689
PSMD11	26S-proteasome-subunit-p44.5	Protein degradation	AB003102
RAF1	c-raf-1	Oncogenesis	X03484

Symbol	Gene	General Function	Gene bank
RB1	Retinoblastome1	regulator of transcription	NM_000321
RRM1	Ribonucleotide-reductase M1	DNA synthesis	NM_001033
S100A	S100 calcium binding protein A4	Cell cycle control	NM_002961
S100A8	Calprotectin	Inflammatory response	NM_002964
S9	Ribosomal Proteine S9	Protein synthesis	NM_001013
SDS	Serine Dehydratase		NM_006843
SHC	p66-SHC transforming protein1	Cell proliferation; apoptosis	U73377
SM22	Transgelin	Muscle development	M95787
SMAD1	SMAD1	Signal transduction	U59423
SMAD2	SMAD2	Signal transduction	U68018
SMAD3	SMAD3	Signal transduction	U68019
SMAD4	SMAD4	Signal transduction	U44378
SOD2	Superoxide dismutase2	Stress response	NM_000636
SPRR1B	Cornifin	Cell structure	NM_003125
TB10	Thymosin beta 10	Cell motility	NM_021103
TBXA2R	Thromboxane-A2-receptor	Respiration	D38081
TERT	Telomerase-reverse transcriptase	DNA synthesis	AF018167
TFAP2A	Transcription factor AP2-alpha	Morphology	M36711
TFAP2B	Transcription factor AP2-beta	Neurogenesis; morphology	X95694
TFAP2C	Transcription factor AP2-gamma	Morphology	NM_003222
TFR	Transferrin receptor	Transporter	NM_003234
TGFBR2	TGF-beta-R2	Cell proliferation	D50683
TIMP1	Tissue inhibitor of metalloproteinase1	Inhibitor of degradation of extracellular matrix	NM_003254
TIMP2	Tissue inhibitor of metalloproteinase2	Inhibitor of degradation of extracellular matrix	NM_003255
TK1	Thymidine-kinase	DNA synthesis	NM_003258
TNFa	Tumor necrosis factor alpha	Apoptosis and proinflammatory response	NM_000594
TOP2	Topoisomerase2-alpha	DNA replication	NM_001067
TPA	Plasminogen activator tissue	Cell migration and tissue remodeling	NM_000930
TRF1	Telomeric repeat binding factor1	Control of mitosis	U40705
TSP1	Thrombospondin 1	Cell adhesion	NM_003246
TSP2	Thrombospondin 2	Cell adhesion	NM_003247
Tubu	Alpha-tubulin	cytoskeletic	NM006082
TYMS	Thymidylate-synthetase	DNA synthesis	NM_001071
UBE2C	Ubiquitin conjugating enzyme E2C/ubiquitin carrier protein	Cell proliferation	NM_007019
uPAR	Urokinase-receptor	Regulation of cell-surface plasminogen activation	NM_002659
VEGF	Vascular endothelial growth factor	Angiogenesis; Cell proliferation	AF022375
VEGFB	Vascular endothelial growth factor B	Angiogenesis; Cell proliferation	U43368
VEGFC	Vascular endothelial growth factor C	Angiogenesis; Cell proliferation	NM_005429
VEGFD	Vascular endothelial growth factor D	Angiogenesis; Cell proliferation	NM_004469
VEGFR1	Vascular endothelial growth factor receptor1	Angiogenesis; Cell proliferation	NM_002019
VEGFR2	Vascular endothelial growth factor receptor2	Angiogenesis; Cell proliferation	NM_002253
VEGFR3	Vascular endothelial growth factor receptor3	Angiogenesis; Cell proliferation	NM_002020
VWF	Factor von willebrand	Role in blood coagulation	NM_000552

Discussion

CHAPTER 9: MODELS OF HUMAN AGEING

The goal of gerontology is to preserve health and well-being, and extent healthy life. We aim to understand the mechanisms of ageing to understand how these mechanisms lead to pathologies (de Grey *et al.*, 2002; de Magalhaes, 2003a). Due to the difficulties in studying human ageing directly, the choice of models employed by researchers is of critical importance. In fact, one of the reasons why the mechanisms responsible for human ageing remain largely a mystery is the lack of appropriate models where scientists can test their hypothesis.

9.1. Lessons from the evolution of ageing

In the first part of this work, we created a model for the evolution of mammalian ageing. Previous theories attempting to explain the evolution of ageing failed to explain several observations, such as animals with negligible senescence--which are deemed impossible to exist by the theory--and why ageing, a phenotype that escaped natural selection, is so similar amongst mammals. It is difficult to reconcile the disposable soma theory (Kirkwood, 1977) with observations of animals whose mortality decreases with age in the wild while reproductive output increases (Congdon *et al.*, 2001). In fact, another recent 38-year field study suggested that older female Painted Turtles, when compared to younger animals, feature increased reproductive output and offspring quality while maintaining survivorship (Congdon *et al.*, 2003). Inter-individual comparisons in humans also failed to support the disposable soma theory (Le Bourg, 2001). In addition, a rethinking of the evolutionary theory of ageing was recently proposed: the hypothesis is that intergenerational transfers--e.g. nurturing--also shape ageing in social species (Lee, 2003). Therefore, it appears that the classical evolutionary theory of ageing does not offer a complete picture on the evolution of ageing across different species.

In contrast to the general view of the classical evolutionary theory of ageing (reviewed in Rose, 1991), our work offers a specific model of how ageing may have evolved in mammals. Importantly, our evolutionary model explains why reptiles feature non-aging organisms and some life-extending traits, such as oocyte regeneration, that are absent even from whales, the longest-lived mammal. In fact, some researchers have found bizarre that mammals feature ageing when more primitive species such as fishes and reptiles appear to avoid it (Hayflick, 1994), while others have wondered why some mammals can be found senescent in the wild (Finch, 1990). Our model explains these observations and contradicts some aspects of the classical evolutionary theory of ageing such as the disposable soma theory. Though we do not

question the validity of, for instance, Medawar's model of mutation accumulation (Medawar, 1952), our model is a step forward in having a more elaborate view of the evolutionary forces shaping ageing, paying special attention to human ageing.

We can expect that even more detailed models of the evolution of ageing in mammals will emerge taking into account, for instance, the evolution of longevity in different mammalian families after the dinosaurs disappeared. For instance, some researchers argue that the slow human life cycle is related to our brain's size and energy requirements (Parker, 1990). Analysing such trends in hominid evolution and ageing may be profitable. Another promising future prospect is the analysis of the evolution of cancer in mammals following the same strategy we employed in developing our model.

The evolution of ageing serves as a theoretical framework to understand the mechanisms of ageing. We used our evolutionary model to suggest that the mechanisms of ageing may be unique in mammals. Certain pathway, such as those involved in metabolism, appear to affect ageing across a variety of organisms (Longo, 1999; Longo and Fabrizio, 2002; Butler *et al.*, 2003; Nystrom, 2003). Yet although the link between metabolic rate and pace of ageing may be widespread, the mechanisms of ageing need not. For instance, caloric restriction delays development (reviewed in Hayflick, 1994), suggesting that shifts in metabolism affect the entire genetic program delaying ageing independently of the mechanisms involved. Furthermore, some pathways that affect ageing in invertebrates do not appear to play a role in mammalian ageing. For instance, the way antioxidant enzymes modulate ageing in invertebrates (Orr and Sohal, 1994) has so far not been extrapolated to mammals (Lipman *et al.*, 1998; Schriner *et al.*, 2000 for arguments). Taken as a whole, these results lead us to suggest that there may be unique mechanisms of mammalian ageing with various consequences in studying human ageing, which will be detailed throughout this chapter 9.

In our model, we predicted that reptiles may feature unique mechanisms to delay ageing and age-related debilitation. Recent findings suggest that turtles have enhanced mechanisms to protect against ROS formation and damage (Lutz *et al.*, 2003). Similarly, studies on cells from turtles suggest that these do not reach RS even in the absence of telomerase (Christiansen *et al.*, unpublished) while other results indicate that at least in some turtles telomere shortening does not occur *in vivo* (Girondot and Garcia, 1999). Moreover, our model may help explain why mammals lost part of their tissue regeneration capacity when compared to, for instance, amphibians and reptiles (Brockes *et al.*, 2001). Taken together, these results support our model for the evolution of mammalian ageing and, as will be discussed in chapters 9.2., 9.4.2., and 9.6, make reptiles an intriguing model to study ageing.

9.2. Animal models

Probably the major conclusion from our work on the evolution of ageing concerns the choice of organisms as models of human ageing. Of course human studies should always be preferred, but often model organisms are unavoidable. If so, then we argue that mammals are the most appropriate models while lower life forms such as yeast or even invertebrates may not be adequate to study the human ageing process. For example, evolutionary distant bacteria have been used to study ageing (reviewed in Nystrom, 2002). Just recently, senescence was shown in a bacterium with asymmetric division, *Caulobacter crescentus*, in the form of a decrease in reproductive output with age (Ackermann *et al.*, 2003). Yet there is no reason to expect a unicellular organism to age for the same reason humans age. For instance, Sir2 has been demonstrated as an important factor in *S. cerevisiae* longevity (Lin *et al.*, 2000) and later in *C. elegans* (Tissenbaum and Guarente, 2001) but findings in *Drosophila* contradict the role of Sir2 in ageing (Newman *et al.*, 2002). Since *Drosophila* are considered a more reliable model of human biology and genetics than yeast or roundworms (Hedges, 2002), then research on Sir2 in yeast may be totally irrelevant for human ageing. Of course studies in lower life forms can help us understand the dynamics and structure of human ageing. If ageing is the corruption of life, then understanding how life itself works may help us understand ageing and much of what we know about life--e.g. DNA replication and repair--came from studies in lower life forms such as bacteria and yeast. Many genes that modulate ageing have been identified in model organisms, namely in invertebrates such as *C. elegans* and *Drosophila* (reviewed in Tower, 2000; Johnson, 2002). These findings can serve as the basis for research into mammals (Tissenbaum and Guarente, 2002; Butler *et al.*, 2003; Warner, 2003), so we do not conclude that research in invertebrates is irrelevant in gerontology. Nonetheless, our work argues that studies on the mechanisms of ageing in lower life forms must be corroborated in mammalian models before extrapolations into human ageing can be made and explaining human ageing based on research in lower life forms is incorrect.

Of course that even eutherians may have different mechanisms of ageing. One excellent example comes from the insulin-signalling pathway, which has been implicated in ageing from invertebrates to mice (Longo and Fabrizio, 2002; Butler *et al.*, 2003). Deletion of the growth hormone receptor gene (*GHR*) in mice increases longevity 40-50% and delays the rate of ageing (Coschigano *et al.*, 2000). Yet human patients with mutations in the *GHR* gene--called Laron syndrome--also exist and, though they have short stature and low levels of insulin-like growth factor I, data obtained so far does not suggest an increased longevity (Zhou *et al.*, 1997;

Kopchick and Laron, 1999). Some studies suggest that insulin-like growth factor I may affect human longevity (Bonafe *et al.*, 2003) and human patients with a mutated *Prop1* gene may live longer (Krzisnik *et al.*, 1999; Bartke *et al.*, 2001a), though no information suggests that humans with this mutation approach the 50% longevity increase seen in mice mutant for *Prop1* (Brown-Borg *et al.*, 1996). Moreover, untreated patients with growth hormone deficiency have a reduced longevity (Besson *et al.*, 2003). Our view is that what occurs in other mammals may not be representative of human biology (reviewed in Davenport, 2003) and, namely, mice may be inadequate to study human ageing (Austad, 1997b for arguments). Therefore, if we base our understanding of human ageing on model organisms, we must have a coherent view of mammalian ageing before we can claim we understand the human ageing process.

In our work, we argued that reptiles age slower than mammals. Clearly, some reptilian taxa feature ageing (Perez-Campo *et al.*, 1993; Patnaik, 1994; Townes-Anderson *et al.*, 1998; Majhi *et al.*, 2000; Jena *et al.*, 2002). In one recent study, lizards that grew faster in early life showed an increased mortality in adulthood (Olsson and Shine, 2002). Yet not only reptiles age slower than size-equivalent mammals, but several reptilian families feature apparently non-ageing animals. Although metabolic rates may also play a role (Castanet, 1994), they do not explain why, for instance, some reptiles feature continuous tooth replacement and often female reptiles feature oocyte regeneration. In addition, the leatherback turtle, *Dermochelys coriacea*, shows signs of endothermy (Davenport *et al.*, 1990) and yet lives at least 30 years (Spotila *et al.*, 2000). We do not argue that all reptiles age slower than all mammals for that would be unrealistic given the flexibility demonstrated by natural selection in modulating longevity and ageing (Cutler, 1979; de Magalhaes, 2003a for arguments). What we argue is that mammals lack certain anti-ageing mechanisms that are present in some or all reptiles. As mentioned in chapter 9.1., pathways unique to reptiles have already been identified that could contribute to the delayed reptilian ageing (Lutz *et al.*, 2003; Christiansen *et al.*, unpublished). For instance, neurogenesis is predominant in reptiles (Font *et al.*, 2001). Other studies found unique traits in reptiles that could be useful to humans: crocodiles have been shown to possess novel antimicrobial peptides (Shaharabany *et al.*, 1999; Fullerton-Smith, 2000). In addition, studying how ageing emerged in some reptiles may shed light on the mechanisms present in mammals. One of the major conclusions from our work is that reptiles are an underestimated model for the study of ageing and more attention should be given to study reptilian ageing or apparent absence of ageing.

Some amphibians and fishes also feature negligible senescence (reviewed in Finch, 1990; Cailliet *et al.*, 2001). Since amphibians and fishes are evolutionary further from us than reptiles, they are not, *a priori*, better models but can still prove useful. For example, amphibians can

regenerate entire limbs while mammalian tissues, such as muscle, can only regenerate as isolated entities (reviewed in Carlson, 2003). Lastly, birds too may be useful models for understanding human ageing because birds are homeotherms featuring high metabolic rates and slow rates of ageing when compared to mammals (Holmes and Austad, 1995 & 2001). In conclusion, we argue that research into ageing should involve models as evolutionary close as possible to humans and a more diverse choice of animal models, including long-lived animals. To understand human mechanisms of ageing we must study humans or mammals, but developing anti-ageing interventions may be based on reptiles and other long-lived animals.

9.3. Human models

As mentioned before, studying the human ageing process has technical difficulties but, if successfully tackled, would prove incredibly valuable. If some genetic factors can delay or accelerate human ageing, then identifying such factors is a powerful approach.

9.3.1. Centenarians

Clearly, there are genetic determinants behind human longevity (Ljungquist *et al.*, 1998; Gudmundsson *et al.*, 2000; Barzilai *et al.*, 2001; Terry *et al.*, 2003). Finding polymorphisms or alleles that correlate with centenarians is not a new strategy in gerontology (Bonafe *et al.*, 2003; Louet *et al.*, 2003). For example, it was shown previously that the different alleles of the *ApoE* gene are distributed differently in centenarians and controls aged 20-70 years (Schachter *et al.*, 1994). Yet *ApoE* was chosen as a candidate gene. Due to our lack of knowledge regarding the human ageing process, candidate genes are presently hard to find. For example, in mice, *klotho* has been associated with premature ageing (Kuro-o *et al.*, 1997). A recent study found a correlation between a variant of the human *klotho* homologue and longevity (Arking *et al.*, 2002), demonstrating how genes found in model organisms may be later studied in humans. Yet with the human genome (Lander *et al.*, 2001; Venter *et al.*, 2001), genome-wide approaches become possible without no *a priori* knowledge about the genes involved. For example, a genome-wide scan found evidence of linkage to human longevity in a locus on chromosome 4 (Puca *et al.*, 2001).

The major drawback of this approach is that it may locate genes that affect longevity without affecting ageing (Perls *et al.*, 2002). For example, long-lived individuals may feature

alleles or polymorphisms that when absent simply increase disease predispositions that decrease longevity.

9.3.2. Werner's syndrome

The phenotype of WS has for long fascinated gerontologists. Even if WS is not real accelerated ageing, the capacity one gene has to modulate so many age-related changes is astonishing. That was one of the reasons why we decided to study SIPS in WS fibroblasts. Although we failed to develop a model of SIPS in WS fibroblasts, our results demonstrate that WS fibroblasts are more sensitive to oxidative stress in the form of H_2O_2. Our results also suggest that the DNA-binding activity of p53 does not increase significantly in WS fibroblasts after a single H_2O_2 stress at 24 or 72 hrs after the stress (de Magalhaes *et al.*, in press), indicating changes in the stress response involving p53. Since the WS fibroblasts grew slowly and showed the morphology of old cells both before and after a single H_2O_2 stress, these results suggest an uncoupling between SA β-gal and the senescent morphogenesis. One possibility is that cells that would normally enter SIPS instead die in WS affecting tissue regeneration. More work is necessary to unravel the mechanisms behind SA β-gal activation and the role of WRN in the phenotype of WS fibroblasts. For instance, ATF-2 and $p38^{MAPK}$ in WS cells should be investigated since these appear to play an important role in inducing SA β-gal (Frippiat *et al.*, 2002). In addition, recent results suggest that WRN plays a structural role in DNA repair independent of its enzymatic activities (Chen *et al.*, 2003b), so studying its interaction partners is a promising avenue. Future experiments could also focus on the senescent phenotype of WS cells aged in culture as well as comparisons between those obtained from young and old patients.

Previously, it was known that p53-mediated apoptosis is attenuated in WS cells (Spillare *et al.*, 1999) and WRN can induce p53 in response to DNA damage (Blander *et al.*, 2000). Together with our results, this suggests changes in the response to DNA damage in WS fibroblasts, which is in accordance with the increased genomic instability of WS cells (Fukuchi *et al.*, 1989). Though further work is necessary, perhaps the increased susceptibility of WS cells to oxidative stress as well as the diminished activation of apoptotic and senescent pathways contributes to the cancer susceptibility of WS patients. In addition, our results suggest that the gene expression patterns of WS fibroblasts are more closely related to young BJ than to senescent BJ HDFs, though protein comparisons suggest that the premature ageing process of WS fibroblasts shares only part of the *in vitro* ageing process of normal fibroblasts (Toda *et al.*, 1998). Gene expression patterns of progeria fibroblasts have also been more closely related

HDFs from old than to young donors, though gene expression during RS has not been previously correlated with progeria fibroblasts (Park *et al.*, 2001). Determining how WRN influences gene expression patterns appears a promising approach and certainly much work will emerge in a near future. Recent results at a gene expression level suggest that WS is indeed accelerated ageing (Kyng *et al.*, 2003).

Of course that WS may be a result of some indirect effect of WRN, as suggested by others (Johnson *et al.*, 1999). It appears certain that WS derives from lack-of-function rather than the toxic effect of an abnormal WRN (Chen *et al.*, 2003b for arguments). Yet, for instance, perhaps mutating *WRN* changes the expression of some other gene that in turn causes the accelerated ageing phenotype. Alternatively, perhaps accelerated ageing in WS and other progeroid syndromes is caused by an increased cell loss due to RS, apoptosis, or deficient cell replacement, leading to the waning of multiple tissues, as proposed previously (Warner and Sierra, 2003). Yet since WS is the pathology that more accurately resembles accelerated ageing, such prospect appears unlikely. In fact, many of the genes involved in progeroid syndromes appear to affect nuclear proteins: most notably Hutchinson-Gilford's syndrome (Eriksson *et al.*, 2003), but also Cockayne Syndrome Type I (Henning *et al.*, 1995). Therefore, it appears likely that proteins affecting DNA metabolism are involved in ageing. The way WS fibroblasts are susceptible to oxidative stress suggests that changes at the DNA level are a causal factor in ageing, as will be detailed in chapter 10.4. Obviously, studying these diseases and the mechanisms involved appears a promising approach to understand the human ageing process. Mouse models may also prove fruitful. For example, a mouse model of Hutchinson-Gilford's syndrome has been recently described (Mounkes *et al.*, 2003).

No disease is a true phenocopy of human ageing and that is why the term "segmental progeria" is often used. In fact, even if certain diseases accelerate age-related pathologies, it should not be assumed that the mechanisms are the same. For instance, the morphology of cataracts in WS differs from senile cataracts (reviewed in Martin, 1978). Therefore, studying progeroid syndromes must be carefully approached. Nevertheless, we think that understanding progeroid syndromes in mouse and man, and how WRN can modulate so many age-related changes should increase our knowledge over normal ageing.

9.4. Cellular models

Although we advocate studies in long-lived turtles and WS, performing such studies *in vivo* has clear limitations. That was why we studied WS fibroblasts instead of WS patients.

Studies like those in Blanding's and Painted Turtles are informative but required decades of field work (Congdon *et al.*, 2001 & 2003). A quicker and cheaper way is to study cells of long-lived animals (Austad, 2001b). That is why cellular studies will continue to be an important approach. Namely, studies involving human cells will continue to be important tools to understand human ageing.

9.4.1. Senescence, RS, and SIPS

As mentioned in chapter 2.4., cells from older donors divide less vigorously (Waters and Walford, 1970; Hayflick, 1994). It is likely that changes occur at a cellular level as the human body ages, but these changes are not as obvious as it may seem at first. For example, studies comparing HDFs from young donors and centenarians failed to find significant differences in terms of cumulative population doublings and growth kinetics (Tesco *et al.*, 1998). On the other hand, recent findings suggest that HDFs from centenarians are less resistant to H_2O_2-induced DNA damage. The same study found conflicting results in terms of the proportion of DNA breaks in centenarians (Chevanne *et al.*, 2003). Similar studies in mice showed that there is a correlation between longevity and oxidative stress resistance of senescent cells (Yegorov and Zelenin, 2003). Studies on small intestine stem cells showed an age-related functional decline in mice (Martin *et al.*, 1998), though extrinsic factors should not be completely discarded. Similar studies found age-related differences in the signalling cascades of mice hepatocytes (Li and Holbrook, 2003). Old stem cells have a diminished function when subjected to stress, suggesting intrinsic changes (reviewed in Van Zant and Liang, 2003). Also, cells transplanted from animal to animal also appear to age and die, suggesting intrinsic mechanisms (reviewed in Hayflick, 1994). Therefore, identifying what changes occur in human cells as we age may prove valuable to understand ageing. For example, understanding why cells become less responsive to external insults and signals may be important to understand human ageing.

Since RS was first discovered by Hayflick and Moorhead (Hayflick and Moorhead, 1961), it has been the subject of much attention. As mentioned in chapter 2.4., the relation between RS and ageing is unclear and several studies suggest that the changes occurring during *in vivo* and *in vitro* ageing are different (Takeda *et al.*, 1992; Chevanne *et al.*, 2003 for arguments). Also mentioned in chapter 2.4., senescent cells can been found *in vivo*, though mostly in situations of pathology (Mendez *et al.*, 1998a; Mendez *et al.*, 1998b; Paradis *et al.*, 2001; Going *et al.*, 2002; Minamino *et al.*, 2002). For instance, one recent *in vivo* study on human muscle found an overexpression of $p21^{WAF1}$ in aged donors (Welle *et al.*, 2001 & 2003)

and another study found increased levels of $p16^{INK4a}$ associated with ageing human kidney (Chkhotua *et al.*, 2003). Cells taken from mice -/- for $p66^{shc}$ have an impaired p53 and $p21^{WAF1}$ stress response (Migliaccio *et al.*, 1999). Whether SIPS or RS are responsible for the appearance of senescent cells *in vivo* is unclear but perhaps apoptosis and cellular senescence play a role in the ageing phenotype (Campisi, 2003 for arguments). Finally, if RS and SIPS are just a tumour protecting mechanism, then the increase in the proportion of senescent cells *in vivo* may be a result and not a cause of ageing.

Mice mutant for the murine *WRN* homologue do not age faster and yet their fibroblasts display hypersensitivity to 4-nitroquinoline-1-oxide and reduced replicative potential (Wang *et al.*, 2000a). This demonstrates how cellular studies may not be representative of organismal ageing, at least in mice. In humans, immortalization of cells taken from patients with WS, Hutchinson-Gilford's syndrome, or ataxia telangiectasia does not fully reverse the phenotype of these cells (Saito and Moises, 1991; Choi *et al.*, 2001; Naka *et al.*, in press). Moreover, cellular studies may be biased because culture conditions may be inaccurate representation of *in vivo* conditions (reviewed in Rubin, 1997). For example, as mentioned in chapter 2.3., 20% O_2 is not representative of physiological conditions (Halliwell, 2003). Recent results show that oxygen sensitivity limits the proliferative capacity of murine fibroblasts (Parrinello *et al.*, 2003). Future experiments must consider O_2 toxicity as an important factor. For example, one future experiment would be to test whether WS cells proliferate differently at lower O_2 concentrations.

In conclusion, it appears that SIPS may be an important mechanism in certain age-related pathologies and stress-prone tissues but there is little evidence that either SIPS or RS occur widely in normal ageing. Based on present information, we do not think SIPS or RS are accurate models of the human ageing process. Cellular senescence may be used as a marker of pathology or even of ageing and to investigate the age-related increase in cancer incidence, but it is unlikely to be a cause of ageing. Therefore, though studies at a cellular level are a worthwhile method to study human ageing, it is dubious which aspects to study. Studying cellular responses to external stressors and signals appears a promising enterprise as these appear to change during the normal human ageing process and in progeroid syndromes such as WS.

9.4.2. Comparative biology

As mentioned earlier, one of the conclusions of our work is that comparative biology may yield important clues about human ageing. To avoid time-consuming and expensive *in vivo* studies, cellular studies may help clarify why different species age at different rates. For

instance, it is clear that intrinsic differences exist between human and murine cells (reviewed in Smogorzewska and de Lange, 2002). In addition, a correlation appears to exist between stress resistance *in vitro* and mammalian longevity (Kapahi *et al.*, 1999) and skin fibroblasts from long-lived dwarf mice are more resistance to a variety of stressors (Murakami *et al.*, 2003). Understanding these differences may prove valuable to understand ageing. For instance, studying SIPS should be extrapolated to other mammalian species.

Based on our work, we predict that experimental studies using cells from long-lived organisms are a potentially important approach to study ageing and cancer, as previously suggested (Austad, 1997a & 2001). Studies using long-lived animals such as Blanding's Turtles (Congdon *et al.*, 2001) or Bowhead whales (George *et al.*, 1999) may prove valuable to our understanding of ageing and cancer. In fact, we tried to obtain cells from both Blanding's Turtles and Bowhead whales by contacting, respectively, Justin Congdon of the Savannah River Ecology Laboratory in Aiken, South Carolina, USA, and John George of the Department of Wildlife Management in Barrow, Alaska, USA. Unfortunately, we failed to obtain the necessary cultures. James Christiansen of Drake University in Des Moines, Iowa, USA, offered to give us immortal Painted Turtle cells--a species with negligible senescence (Congdon *et al.*, 2003)--, but we gave up on reptilian cells since experimental conditions (Rund *et al.*, 1998) would be impossible to obtain in our laboratory in time to start any relevant experiment.

One major problem with cellular studies involving different species is that culture conditions may vary. For instance, reptilian cells cannot be cultured for long times at 37°C (Goldstein, 1974; Rund *et al.*, 1998). Changes in culture parameters may bias comparisons between different cell lines. Even so, comparisons between, for instance, different mammalian cell lines may prove relevant to understand mammalian ageing and consequently human ageing. In fact, for instance, studies of telomere biology in non-human primates are already underway (Steinert *et al.*, 2002). We anticipate this is an area with much potential.

9.5. Computer models

Computer models of RS have been built based on extensive computer simulations (Kitano and Imai, 1998) and many others have suggested computational approaches to study ageing (Hood, 2003; Kirkwood *et al.*, 2003). As will be detailed in chapter 11, we also published a strategy to understand human ageing based on computational approaches: we described the modern computational tools in the context of gerontology, providing ideas on how to apply computer models to study human ageing. Overall, our proposal is that, due to the difficulties in

studying human ageing, researchers aim to uncover the structure of the human ageing process, as well as find key controlling nodes which can eventually be developed into a Boolean model of human ageing.

We think high-throughput computational methodologies will play a crucial role in understanding human ageing. One promising approach involves DNA microarrays, which may be applied to mammals or even humans. If the amount of data generated is large enough, it may be possible to interpret cross-sectional human studies and extract valuable information. Indeed, gene expression results have been obtained by comparing young and old samples of human skin (Ly *et al.*, 2000), retina (Yoshida *et al.*, 2002), and muscle (Welle *et al.*, 2001 & 2003), and proteomic studies on brain ageing have already been undertaken (Chen *et al.*, 2003c). Gene expression results have also suggested that WS is accelerated normal ageing (Kyng *et al.*, 2003). Although many genomic features remain obscure and could play a role in ageing, we think transcriptional regulation is the key to understand how the ageing process is controlled in mammals, as suggested by others (Martin, 1978; Cutler, 1979; Roy *et al.*, 2002; Hood, 2003). Based on the likelihood that transcriptional regulation plays an important role in determining mammalian rate of ageing, we defend that transcriptional studies will play an important role in the future. Potentially powerful techniques involve the study of transcription either through pure computational methods, such as transcription factor binding site predictions, or by coupling DNA microarray data to computational techniques.

In accordance with our theoretical framework, we developed a computational toolkit to serve as an example of our strategy: Ageing Research Computational Tools (ARCT). ARCT is based on our previous work on evolution that led us to advocate comparative biology as a powerful approach to understand ageing. Studies involving phylogenetic profiles, functional interactions, and transcriptional regulation are made possible through our toolkit (de Magalhaes and Toussaint, submitted for publication; Appendix). ARCT is available online at: http://genomics.senescence.info/software/

We applied computational approaches to the data obtained using the DualChip Human General. For instance, we tried to determine whether TFBS detection could help explain our gene expression results through the detection of putative p53 and HIF-1 binding sites. Unfortunately, our results did not show any significant trends. We also failed to find any chromosomal clustering from our genes differentially expressed in young and old BJ HDFs (Figure 25). Since another recent paper found such clusters in BJ HDFs (Zhang *et al.*, 2003), we can only assume that the relatively small amount of genes (i.e. 202) we studied by the DualChip Human General does not allow us to estimate chromosomal clustering.

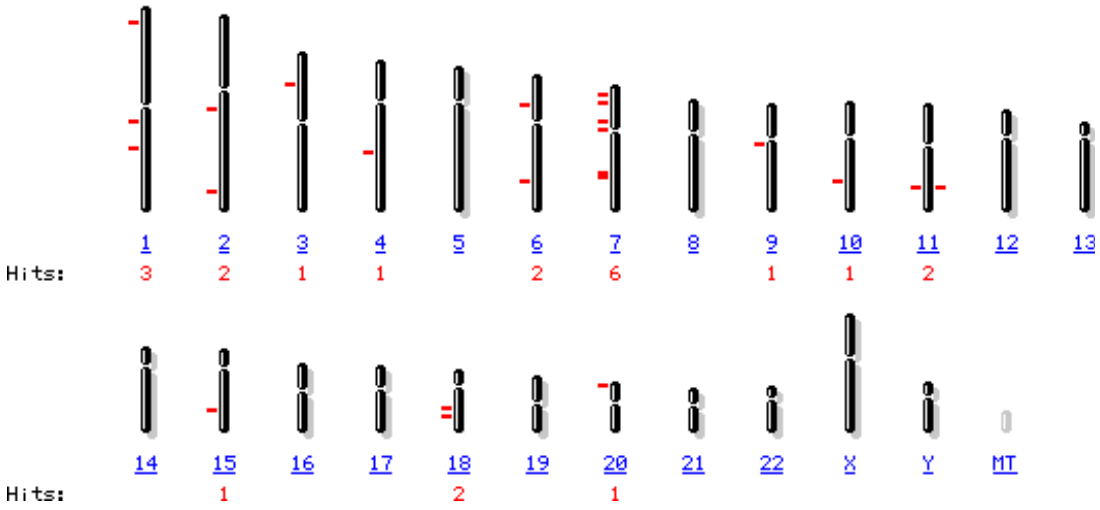


Figure 25: Chromosomal location of genes upregulated in BJ HDFs at late cumulative population doublings when compared to young controls (Table 5 of article 3 in chapter 8). Overall expression patterns do not reveal any significant chromosomal clustering. The clustering in chromosome 7 is due to transcriptional variants of the same gene. Calculated using the Ageing Research Computational Tools 0.7beta through build 34 of the *Homo sapiens* genome at the NCBI's Map Viewer: http://www.ncbi.nlm.nih.gov/mapview/

9.6. Developing anti-ageing therapies

As emphasized throughout this work, the ultimate goal of gerontology is to understand human ageing to enhance health and preserve life. In fact, the gerontological community should re-assess its goals as understanding the basic ageing process should be a much higher priority than understanding age-related diseases, as argued by others (Adelman, 1998; Hayflick, 2000). Unfortunately, at present, there is no proven way to delay human ageing (reviewed in Olshansky *et al.*, 2002). Although neglected for a long time, the possibility of developing true anti-ageing therapies is now within our reach (de Grey, 2003a; de Magalhaes, 2003b). Our definition of anti-ageing therapies does not involve interventions that solely extend longevity but rather interventions that delay the ageing process by, for instance, delaying the rate of ageing. The most useful method to detect changes in rate of ageing is to calculate the rate at which mortality increases with age (Kowald, 2002 for arguments). Although other methods may be more accurate, due to common restrictions on sample size, the Gompertz equation remains the most accurate way to determine rate of ageing.

If chromatin changes play a role in ageing, as will be detailed in chapter 10.4., then anti-ageing therapies may involve chromatin remodelling factors. One study showed that feeding sodium 4-phenylbutyrate (PBA) to *Drosophila* extends longevity by about 40% (Kang *et al.*, 2002). PBA is a histone deacetylase inhibitor, which induces hyperacetylation of histones resulting in a relaxing of the binding of histones to chromatin that in turn affects gene transcription (Lea and Randolph, 1998). Of course these results must be interpreted carefully. PBA was initially used in humans to treat urea cycle disorder (Brusilow *et al.*, 1984) and it has been used to treat several human diseases (reviewed in Kang *et al.*, 2002). Even so, it may be possible to create drugs that target specific chromatin modifications. Epigenomics is a growing science (reviewed in Novik *et al.*, 2002) and some evidence indicates that methylation is involved in human age-related degeneration (Post *et al.*, 1999). Epigenetic events are crucial in cancer (Jones and Baylin, 2002). Therefore, this is an area with much promise and, for instance, microarray analysis of DNA methylation is becoming a reality (Shi *et al.*, 2003). It is important to clarify that although genetic mechanisms may regulate ageing (de Magalhaes, 2003a), fixing those mechanisms should not be as simple as fixing the telomeres in RS.

In our work, we suggested that instead of identifying changes as people grow old, it may be relevant to identify changes that occur during development that allow ageing to commence, as will be detailed in chapter 11. If ageing and development are two distinct processes, as it might be the case (Miller, 1999 for arguments), then identifying the changes that occur prior to, for

example, puberty, may prove valuable to develop anti-ageing interventions. One possibility, for instance, are mtDNA mutations accumulating from early in life (Khrapko *et al.*, 2003). Development and ageing, however, may be connected (Parker, 1990 for arguments).

One ongoing approach is to identify potential therapeutic targets in model organisms (Rose *et al.*, 2002b for arguments). In a recent example, a study identified several molecules that extend longevity in *S. cerevisiae* and suggested them as of potential therapeutic interest against human ageing (Howitz *et al.*, 2003). Our work, of course, argues against such approaches and deems such conclusions as imprudent. For instance, as mentioned in chapter 9.1., the impact of antioxidants in invertebrate longevity has not been reproduced in mammals and, as will be detailed in chapter 9.6.1., telomerase modulates ageing differently across different species. Although testing interventions to delay ageing in animal models may be inavoidable (Warner *et al.*, 2000 for arguments), it is important to keep a sceptical open mind.

As mentioned in chapter 1.3.2., several attempts have been made to delay ageing through antioxidants. Even though the free radical theory of ageing is unproven, much research is conducted to develop anti-ageing dietary supplements involving antioxidants. Although it is possible to decrease oxidative damage in mice by feeding them acetyl-L-carnitine and/or R-alpha-lipoic acid (Hagen *et al.*, 2002; Liu *et al.*, 2002a & 2002b), there is no evidence that feeding mice antioxidants increases longevity or delays rate of ageing (Lipman *et al.*, 1998 for arguments). On the other hand, $p66^{shc}$ shows that oxidative stress may affect apoptosis and longevity in mice (Migliaccio *et al.*, 1999). Intriguingly, the forkhead transcription factor FOXO3a has been recently shown to protect quiescent cells from apoptosis by overexpressing mitochondrial superoxide dismutase and increasing oxidative defences (Kops *et al.*, 2002). Some evidence indicates that $p66^{shc}$ is somewhat related to the forkhead transcription factor family (Nemoto and Finkel, 2002; Purdom and Chen, 2003a, 2003b), which in turn has been associated with extended longevity in lower life forms (Lin *et al.*, 1997; Honda and Honda, 1999; McElwee *et al.*, 2003). Perhaps upregulating oxidative defences might be a more promising approach. Indeed, some results from mice indicate that overexpression of M1B, a forkhead box transcription factor, can prevent age-related proliferation defects in regenerating liver (Wang *et al.*, 2001).

Another ongoing study involves the search for caloric restriction mimetics in primates. The idea is to identify compounds that replicate the effects of caloric restriction without having to eat less (reviewed in Lane *et al.*, 2002a & 2002b). The fact that this study was initiated in 1987 illustrates one of the greatest problems in developing anti-ageing therapies: the long time it takes to test anti-ageing products (Davenport and Toy, 2002). Obviously, human studies based

on rate of ageing are impossible and model organisms may be inaccurate. One possibility is to test anti-ageing therapies in specific age-related pathologies. Despite its medical interest, this is a biased approach because an alleviation of an age-related pathology may not be a result of a delayed ageing process. The most promising possibility is to develop biomarkers of ageing that can accurately predict how aged someone is, perhaps in combination with caloric restriction and primate studies, as proposed by others (Ingram *et al.*, 2001). DNA microarrays are one promising approach.

Based on our work, mammalian models and long-lived species are preferred over lower life forms. One intrinsic problem in the study of ageing is that models that live less than humans are preferred. If we aim to develop anti-ageing therapies we must learn from other species' virtues, not mistakes. As mentioned in chapter 9.2., understanding the unique mechanisms behind reptilian neurogenesis and oocyte regeneration may be helpful to humans. Similarly, anti-ageing therapies based on long-lived species such as certain reptiles, whales, and birds may be a promising approach. As pointed by others, studying long-lived animals is a much better way to develop anti-ageing therapies than short-lived animals because long-lived animals may have already obviated some deleterious processes still present in short-lived ones (Strehler, 1986). Research is already underway to understand tissue regeneration in adult urodele amphibians to promote mammalian regeneration (Brockes and Kumar, 2002). Cancer research may also benefit from studies on whales (Austad, 1997a). For example, in accordance to what we argued in chapter 9.4., understanding stress response mechanisms, such as the response to DNA damage, may allow us to develop novel ways to preserve the viability of cells, such as stem cells.

In fact, several studies suggest stem cells as of great potential in anti-ageing research (reviewed in Lanza *et al.*, 1999a, 1999b; de Grey, 2003c). For example, ageing has been linked to an age-related inability of stem cells to replenish mature cells and therapeutic interventions that enhance stem cell functional capacity might ameliorate many of the age-associated atrophies in several organ systems (Donehower, 2002). Even the brain degeneration may benefit from stem cells (Le Belle and Svendsen, 2002). With nuclear transfer experiments such as *Dolly* (Wilmut *et al.*, 1997), it is now possible to generate embryonic stem cells from adult individuals that can be used to treat age-related diseases (Cibelli *et al.*, 2002). Therefore, stem cells remain one of the most promising avenues for anti-ageing research.

9.6.1. Telomerase alters the normal cellular functions

The telomeres are the perfect example of our reserves towards employing distant models to study ageing. Telomere dysfunction in *S. cerevisiae* leads to senescence (Lunblad and Szostak, 1989; Lowell and Pillus, 1998). Yet, as mentioned in chapter 3.3., telomerase-negative mice are normal up to four generations (Rudolph *et al.*, 2001) and telomerase overexpression does not alter ageing in mice (Artandi *et al.*, 2002). On the other hand, telomerase dysfunction in humans causes dyskeratosis congenita (Vulliamy *et al.*, 2001). It is therefore nearly impossible to determine whether telomerase is of potential anti-ageing interest based on model organisms and so we will focus on what we know about telomerase and human ageing.

The importance of the telomeres in RS and the ability of telomerase to immortalize human cells led to the suggestion that telomerase can be used as an anti-ageing therapy (reviewed in Fossel, 1996). As mentioned in chapter 3, hTERT expression immortalizes most, though probably not all (Halvorsen *et al.*, 2000; Di Donna *et al.*, 2003), human cell types (Bodnar *et al.*, 1998). Culture conditions with low levels of O_2 or $p16^{INK4a}$ disruption are often necessary for cellular immortalization (Kiyono *et al.*, 1998; Forsyth *et al.*, 2003), suggesting that either telomerase is not sufficient for cellular immortalization or inadequate culture conditions may prevent immortalization of certain cell lines, as will be mentioned in chapter 10.2. Even so, the principle is that if telomerase can prevent RS, it may also prevent cellular ageing *in vivo*. One study found that the telomeric repair efficiency is lower in cells from an old than in cells from a young donor; and a slightly lower efficiency was also reported in WS cells (Kruk *et al.*, 1995). In addition, a recent study found a correlation between telomere length and mortality in people over 60 years of age (Cawthon *et al.*, 2003). As such, telomere dysfunction may play a role in age-related debilitation.

Previously, experimental evidence raised questions on whether telomerase is likely to become a source of anti-ageing therapies (Vaziri *et al.*, 1999; Wang *et al.*, 2000b). Our gene expression results support these views and show considerable changes due to ectopic hTERT expression as well as alterations in the DNA-binding activities of ATF-2 and NF-κB. Interestingly, mitochondrial superoxide dismutase, which was the gene most down-regulated in our study, has been recently hinted as a tumour suppressor (Plymate *et al.*, 2003). Confirming previous reports that telomerase favours tumorogenesis by a telomere length-independent mechanism (Stewart *et al.*, 2002), a recent study found that hTERT expression in HDFs leads to an upregulation of epiregulin, a potent growth factor involved in tumorogenesis (Lindvall *et al.*, 2003). Another recent study found that telomerase modulates the expression of growth-

controlling genes to enhance cellular proliferation (Smith *et al.*, 2003), as also indicated by our results. Taken together, these results suggest that hTERT-immortalized cells are not functionally equivalent to normal cells and using hTERT for therapeutic purposes must be approached with caution. On the other hand, telomerase appears a promising target to fight cancer and indeed several recent proposals have been made to develop anti-telomerase treatments (Hahn *et al.*, 1999; Shammas *et al.*, 1999; Shay and Wright, 2002; Asai *et al.*, 2003; Huard and Autexier, 2003; Nguyen *et al.*, 2003; Saretzski, 2003). Another possibility is using a transient telomerase activation in certain diseases--being dyskeratosis congenita the most obvious example--or cell lines with telomerase expression stringently controlled (Effros, 2003 for arguments).

One possibility is that the differences in gene expression witnessed between BJ and hTERT-BJ1 HDFs derive from the transfection methodology rather than hTERT activity. We do not think this is the case for several reasons. Firstly, the initial reports describing the hTERT-BJ1 cells line indicated that hTERT transfection into BJ cells did not alter normal cellular functions. Transfection of hTERT into other cell lines and comparisons with "vector only" clones were also performed (Bodnar et al., 1998; Jiang et al., 1999; Morales et al., 1999). Secondly, our results suggesting that telomerase favours tumorogenesis by telomere-independent mechanisms has been reported in other cell lines and models, including some overlap with our gene expression results (Oh *et al.*, 2001; Stampfer *et al.*, 2001; Stewart *et al.*, 2002; Lindvall *et al.*, 2003; Smith *et al.*, 2003). Taken together, these results suggest that telomerase activity promotes tumorogenesis independently of telomere elongation.

It is unproven that telomerase can be used as an anti-ageing therapy. For instance, some evidence suggests that hTERT transient expression can occur in human cell lines when necessary for regeneration (Osanai *et al.*, 2002), and there is little evidence to suggest that further hTERT expression is necessary in human tissues (Stephens *et al.*, 2003 for arguments). Importantly, telomerase may alter the normal cellular functions and promote cancer. Therefore, in conclusion, we argue that telomerase is unlikely to become a source of anti-ageing therapies.

CHAPTER 10: DNA DAMAGE, AGEING, AND SIPS

One of the main objectives of our work was to establish the role of the telomeres in SIPS. Since, unlike genomic damage, damage to the telomeres in normal HDFs cannot be repaired, telomere shortening may result in single-strand DNA that would in turn trigger SIPS through the p53 pathway (reviewed in von Zglinicki, 1998). Our strategy was to employ a telomerase-immortalized cell line, hTERT-BJ1, to investigate the mechanisms involved in SIPS. Since in hTERT-BJ1 HDFs the telomeres can be repaired, we hoped to find clues about the role of telomeres in SIPS.

10.1. Critical telomere shortening is not necessary for SIPS

Based on the similar results we obtained in BJ and hTERT-BJ1 cells under SIPS, one of our conclusions was that the telomeres do not play a major role in causing SIPS. Telomere shortening did not reach critical lengths with stress. Previous findings also failed to find a relevant telomere shortening following H_2O_2 (Chen *et al.*, 2001b) or t-BHP stress (Dumont *et al.*, 2001). Radiation-induced senescence was also telomere-independent and SA β-gal positive cells did not show shorter telomeres (Suzuki *et al.*, 2001b). In support of our results, two other studies showed that telomerase-immortalized normal HDFs can reach senescence without telomeres reaching critical lengths (Gorbunova *et al.*, 2002; Matuoka and Chen, 2002). The increased telomere shortening, though probably not solely a result of compensatory cycling, may derive from damage to the telomeres, but it is unlikely that such low amounts of telomere shortening are responsible for SIPS. One hypothesis is that a small amount of telomere shortening disrupts the telomeres or is a signal of telomere dysfunction, but such conclusions are a fruit of speculation.

Although it has been proposed that the telomeres are gatekeepers of the genome (von Zglinicki, 2002), there is no *a priori* reason to expect the telomeres to be different from the rest of the genome in sensing DNA damage. Single strand regions appeared to accumulate more often in the telomeres than in other regions of the genome (Petersen *et al.*, 1998), but other results suggested that double-strand breaks mediate cytotoxicity following oxidative stress such as H_2O_2 (Cantoni *et al.*, 1996; Cantoni and Giancomoni, 1997). Other results also argued that cell death from H_2O_2 does not derive from specific single strand breaks but rather from multiple damage sites to the DNA (Ward *et al.*, 1985). Therefore, no evidence exists that specific damage to the telomeres is the cause of SIPS. One hypothesis is that disruption of the telomeres rather than telomere shortening *per se* is involved in SIPS. As mentioned in chapter 4.2., the

introduction of telomeric oligonucleotides into HDFs induced growth inhibition based on the p53/p21^{WAF1} pathway (Saretzki *et al.*, 1999). Recent results also show that exposure to 40 μM of oligonucleotides homologous to the telomere 3'-overhang sequence induces a senescent phenotype. Though the concentration of oligonucleotides used may be biologically unrealistic, one proposal is that telomere shortening or DNA damage may disrupt the telomere loop causing the exposure of the 3'-overhand and triggering senescence through a DNA-damage response (Li *et al.*, 2003). In addition, other players may be involved in SIPS such as TRF1 and TRF2 (Smogorzewska *et al.*, 2000), and TRF1's interacting partners PINX1 (Zhou and Lu, 2001), POT1 (Loayza and de Lange, 2003), tankyrase (Smith and de Lange, 2000), and TIN2 (Kim *et al.*, 1999). For example, some evidence suggests that TIN2 alters the conformation of TRF1, which favours a tertiary telomeric structure that hinders telomerase from gaining access to telomeres (Kim *et al.*, 2003). Although there is presently no evidence to suggest any of these proteins may play a role in SIPS, since we lack a detailed vision of telomere regulation, further research in this area will certainly give us a better view of the mechanisms involved. Future experimental perspectives should include telomeric proteins in addition to telomere length.

Based on our work, however, genotoxic damage should be considered the main cause of SIPS. Telomere damage recruits telomerase which stabilizes the telomeres (Blackburn, 2000) and telomerase associates with human telomeres enhancing genomic stability and DNA repair (Sharma *et al.*, 2003). One possible mechanism involves the cooperation between telomerase and Ku, a protein involved in DNA repair (Chai *et al.*, 2002). A recent study found that overexpression of hTERT's C-terminal polypeptide sensitized HeLa tumour cells to H_2O_2-stress without affecting hTERT's activity or telomere length. Though not fully understood, these results suggest that hTERT plays a role in mediating resistance to oxidative stress (Huang *et al.*, 2003). Yet our results do not suggest any beneficial effect of hTERT expression in SIPS, contradicting the hypothesis that telomeres play a key role in SIPS. Other results suggest similar conclusions (Matuoka and Chen, 2002), despite a possible protection against apoptosis and necrosis conferred by hTERT (Gorbunova *et al.*, 2002). Perhaps stabilizing the telomeres offers protection against apoptosis, but not against SIPS, suggesting that SIPS does not derive from the telomere-based signalling cascade. In fact, other recent results suggest that high hTERT expression can, in some cases, induce a senescent-like growth arrest in HDFs (Gorbunova *et al.*, 2003), so a telomere-independent senescence-inducing mechanism exists, as will be detailed in the next chapter. Generalized DNA damage generated by H_2O_2 is therefore the mechanism predominant in SIPS. This is one of the major conclusions of our work.

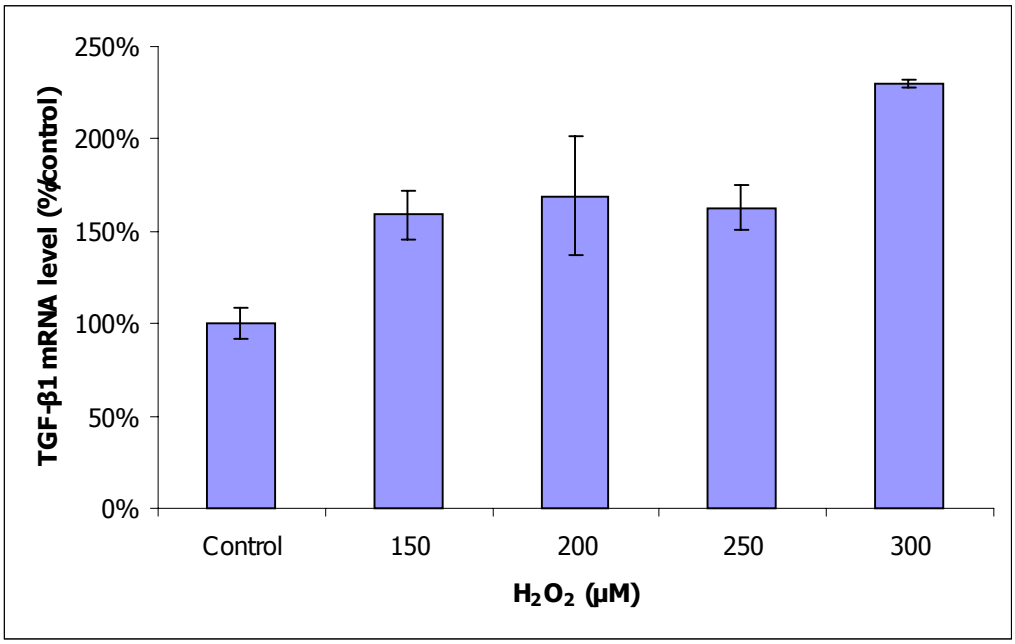
10.2. DNA damage induces cellular senescence through complementary pathways

An important part of our work was to study the mechanisms involved in SIPS. Since hTERT-BJ1 and control BJ HDFs did not appear to feature unique mechanisms, we interpreted the results obtained for both cell lines in conjunction.

Working with Christophe Frippiat, we found that the levels of TGF-β1 increase in AG04431 human skin fibroblasts exposed to H_2O_2 (Frippiat *et al.*, 2001; Figure 26A). Yet our results in BJ cells do not support a role for TGF-β1 in SIPS, at least in our model (Figure 26B). The lack of upregulation by stress of ATF-2's DNA-binding activity supports the view that TGF-β1 is not involved in SIPS of BJ cells, unlike the results shown in IMR-90 HDFs (Frippiat *et al.*, 2002). Since TGF-β appears to regulate DNA synthesis differently in sparse and non-confluent cultures (Stathakos *et al.*, 1993), one hypothesis is that vigorously dividing HDFs, such as BJ HDFs, react differently to TGF-β. A future possibility would be to study TGF-β1 in sparse BJ and hTERT-BJ1 HDFs rather than confluent cultures. Even so, we do not think TGF-β1 is a major player in SIPS of BJ or hTERT-BJ1 HDFs.

Our proposed mechanism involves the p53/$p21^{WAF1}$ pathway. p53 has been previously implicated in SIPS but since its protein level under SIPS returns to control level 48 hrs after a single subcytotoxic H_2O_2 stress, its role has been downplayed (Chen *et al.*, 1998 & 2000b). Although we did not find a significant increase of p53's protein level after subcytotoxic H_2O_2 stress, we found a sustained, marked increase of p53's DNA-binding activity, which is a novel finding. Evidence suggests that post-translational modifications are the molecular basis for p53 activation (Brooks and Gu, 2003), and activate p53 during cellular senescence (Atadja *et al.*, 1995) and in response to stress (Colman *et al.*, 2000), thus corroborating our results and suggesting that p53's role in SIPS has been underestimated. Although preliminary, our results from WS fibroblasts also suggest a role for p53 in SIPS since p53's DNA-binding activity was only slightly increased in WS fibroblasts that did not endure an increase in SA β-gal activity.

Activation of $p21^{WAF1}$ independently of p53 has been reported (Tahara *et al.*, 1995), but $p21^{WAF1}$'s activation in response to DNA damage is likely to be p53-mediated (Michieli *et al.*, 1994). For example, radiation-induced senescence appears to be dependent on p53 (Suzuki *et al.*, 2001b). Though we cannot discard that other players may be involved, in BJ HDFs SIPS is regulated by the p53/$p21^{WAF1}$ pathway which in turn affects pRb's phosphorylation status. A recent study found that p53-mediated senescence can occur via, so far unknown, factor(s) other than $p21^{WAF1}$, suggesting we still lack a complete picture of the pathways involved (Wyllie *et al.*, 2003). Other recent results also demonstrate $p21^{WAF-1}$-independent growth arrest and suggest that



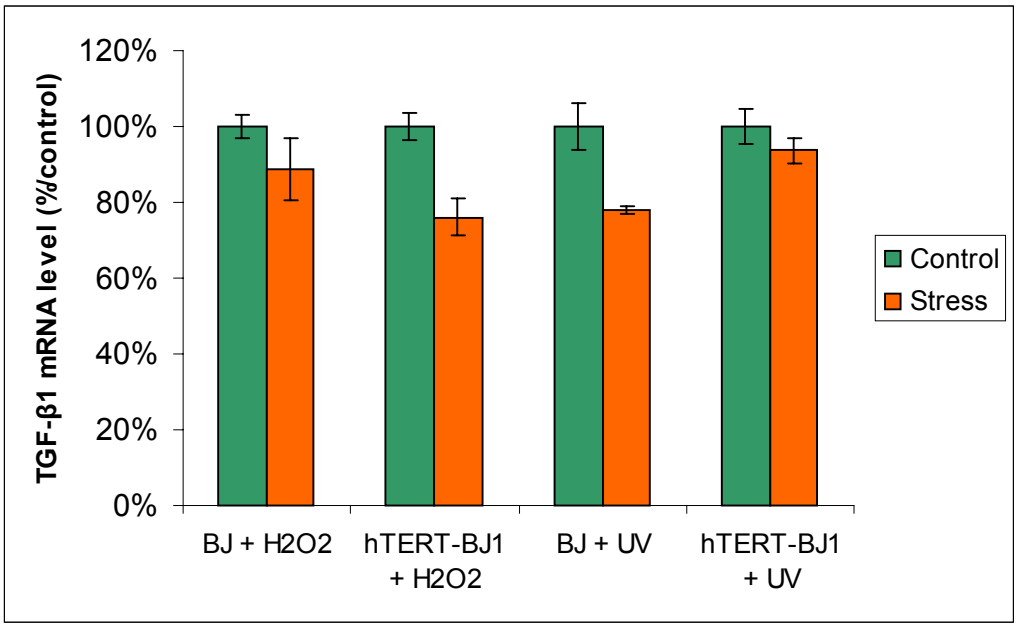


Figure 26: Steady-state level of TGF-β1 mRNA at different doses of H_2O_2 stress in AG04431 HDFs (A). Steady-state level of TGF-β1 mRNA after a single H_2O_2 stress or five repeated UVB stresses in BJ and hTERT-BJ1 HDFs (B). According to the *t*-test, the probability of both or either stresses decreasing the expression of TGF-β1 is above the common threshold of 5% and so we conclude that TGF-β1 mRNA levels do not change with UVB or H_2O_2 subcytotoxic stress in BJ and hTERT-BJ1 HDFs. mRNA was extracted at 48 hr after stress. The results are presented as mean values ± S.D. from three independent experiments. mRNA extraction and RT-PCR conditions according to previously described protocols (Frippiat *et al.*, 2001).

$p21^{WAF-1}$ degradation via ATR is in fact essential for correct DNA repair following low doses of UV irradiation (Bendjennat *et al.*, 2003). Intriguingly, other results from our laboratory indicate that the kinase activity of CDK2 can be greatly decreased in a $p21^{WAF-1}$-independent manner in IMR-90 HDFs (Frippiat *et al.* 2003).

Based on p53's importance, we tried to use antisense oligonucleotides against p53 (Biognostik, Göttingen, Germany) but these failed to diminish p53's DNA-binding activity and thus we decided to abandon this approach (Figure 27). Perhaps studies using interference RNA may be conducted in the future. IMR-90 HDFs expressing E6, and thus lacking p53 activity, are still able to develop a senescent phenotype after subcytotoxic H_2O_2 stress involving a slight activation of $p21^{WAF1}$ and pRb hypophosphorylation (Chen *et al.*, 1998 & 2000b). This suggested that p53 is not essential in SIPS and complementary pathways exist in regulating pRb. Further work is necessary to elucidate these mechanisms.

One recent report argued that HDFs with both the $p16^{INK4a}$ and p53/telomere pathways are more sensitive to SIPS (Itahana *et al.*, 2003). Given the relatively high resistance to H_2O_2 of BJ HDFs, and since the $p16^{INK4a}$ pathway is present in BJ HFDs but inactive during RS (Beausejour *et al.*, 2003), our results support the idea that cell lines without an activated $p16^{INK4a}$ pathway are more resistance to SIPS. Since hTERT expression did not change stress resistance, we cannot corroborate the idea that ablation of the telomere pathway increases resistance to stress.

In fact, our results also cast some doubts on the role of $p16^{INK4a}$ in SIPS. Other results had already suggested that $p16^{INK4a}$ is not overexpressed in senescent BJ cells (Shelton *et al.*, 1999) or following H_2O_2 stress (Chen *et al.*, 2001b). One possible explanation is that $p16^{INK4a}$ is involved in maintaining growth arrest but does not participate in triggering SIPS. For example, oncogenic *ras* can induce $p16^{INK4a}$ in BJ HDFs, showing that the $p16^{INK4a}$ pathway is present in BJ HFDs just inactive during RS (Beausejour *et al.*, 2003). One hypothesis is that the increased resistance to oxidative stress in BJ HDFs avoids $p16^{INK4a}$ activation.

Contrary to what happens in BJ HDFs, $p16^{INK4a}$ is widely overexpressed in pre-senescent and senescent WI-38 HDFs with relatively long telomeres (Beausejour *et al.*, 2003), suggesting that telomere shortening and p53 do not cause RS in WI-38 HDFs. Instead, perhaps RS in WI-38 HDFs is a response to stress or inappropriate culture conditions. One hypothesis is that p53 triggers telomere-driven RS while $p16^{INK4a}$ triggers senescence in response to certain forms of damage and signals--e.g. oncogenic signals or changes in intra-cellular oxidative potential. Recent results show that oxygen sensitivity limits the proliferative capacity of murine fibroblasts (Parrinello *et al.*, 2003). In addition, knocking-out cytoplasmic superoxide dismutase induces

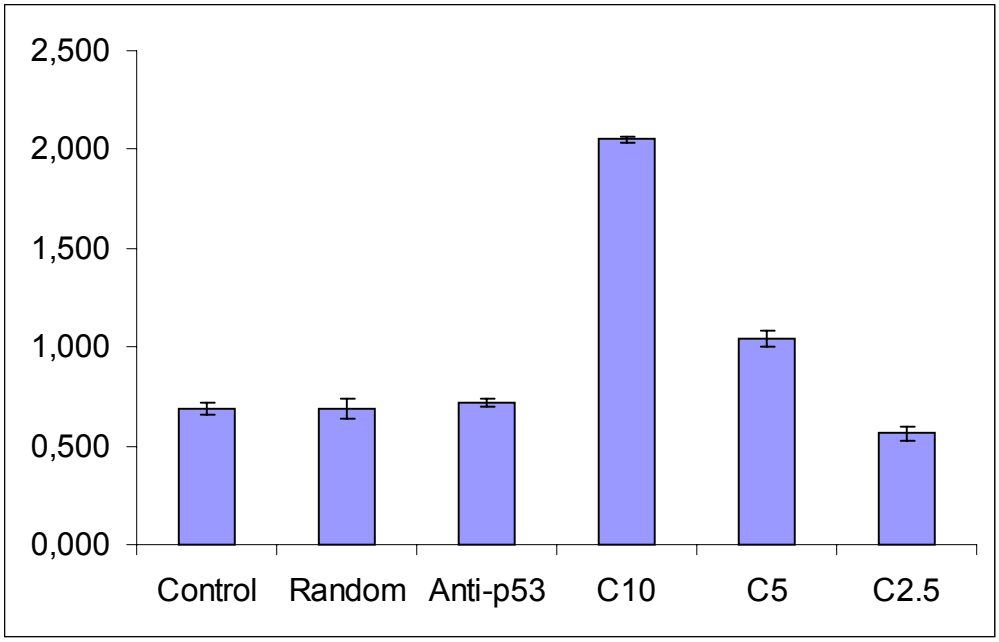


Figure 27: DNA-binding activity of p53 in BJ HDFs. Control indicates cells incubated without oligonucleotides. Random means random oligonucleotides were added for 24 hrs to the medium with a final concentration of 10 μM. Anti-p53 means p53 antisense oligonucleotides were added to the medium for a final concentration of 10 μM for 24 hrs. C10-2.5 is a control sample with various amounts of protein to test the sensitivity of the kit. To determine the DNA-binding activity of p53 we used the TransAM kit (ActiveMotif, San Diego, CA, USA), according to manufacturer's instructions. The results are presented as mean values ± S.D. We had previously demonstrated by fluorescent microscopy that it took less than 8 hrs for oligonucleotides to incorporate into BJ and hTERT-BJ1 HDFs and so concluded that the antisense p53 was not inhibiting p53's DNA-binding activity.

senescence in HDFs (Blander *et al.*, 2003). Oxygen sensitivity affects WI-38 HDFs immortalization with hTERT (Forsyth *et al.*, 2003). Evidence also suggests that epithelial cells reach senescence due to $p16^{INK4a}$, not short telomeres: if $p16^{INK4a}$ is inactivated, the proliferative capacity of epithelial cells is increased and senescence is only reached when telomeres are critically short (Romanov *et al.*, 2001). Perhaps RS results from either telomere shortening or telomere-independent mechanisms such as inadequate culture conditions (Ramirez *et al.*, 2001); therefore, for example, epithelial, WI-38, and WS cells may reach senescence not due to short telomeres but due to their increased sensitivity to O_2. In contrast, BJ cells, which are more resistant to oxidative stress (Lorenz *et al.*, 2001), reach RS due to critically short telomeres. We speculate that cellular senescence evolved as an anti-cancer mechanism to prevent uncontrollable cellular growth, DNA damage, or other oncogenic signals. In fact, HeLa immortal tumour cells may also reach senescence with long telomeres and high telomerase activity if the p53 and pRb pathways are restored (Goodwin and DiMaio, 2001).

Recent results also suggest that $p16^{INK4a}$ may prevent reversal of senescence by activating pRb which in turn changes the chromatin structure (Morrison *et al.*, 2002; Narita *et al.*, 2003). p53 inactivation reverses senescence in some but not all HDFs. $p16^{INK4a}$ suppression confers sensibility to p53 inactivation leading to a reversal of senescence. Recent results also suggest that $p16^{INK4a}$ may act independently of pRb. Lastly, hTERT does not reverse senescence in HDFs, perhaps due to lack of proliferation (Beausejour *et al.*, 2003).

The interaction between apoptotic and senescent pathways has been demonstrated and is probably antagonistic (reviewed in Wang *et al.*, 2003). For example, senescent HDFs are resistant to apoptosis (Wang, 1995) and $p21^{WAF1}$ is cleaved during apoptosis to shift cancer cells from growth arrest to apoptosis (Park *et al.*, 1998; Zhang *et al.*, 1999). In our work, we proposed a rearrangement of apoptotic/anti-proliferative pathways as opposed to anti-apoptotic/proliferative pathways in SIPS. A key player is p53. Although the cascade of events is unclear, activation of p53 by oxidative stress can induce growth arrest--to prevent the replication of damaged DNA--or apoptosis--to eliminate defective cells (reviewed in Sionov and Haupt, 1999; Martindale and Holbrook, 2002; Oren, 2003). The proportion of apoptotic versus senescent cells is dose dependent (Chen and Ames, 1994; Chen *et al.*, 2000b; Toussaint *et al.*, 2002a). Probably the amount of damage endured by cells determines whether cells should enter apoptosis--if the damage is too high--or growth arrest--if the damage is lower, so it can be repaired. Alternatively, perhaps oxidative stress activates apoptotic pathways due to the role of ROS as intra-cellular signalling molecules (reviewed in Green and Reed, 1998). Since even at

subcytotoxic levels a cell population is not homogeneous, under subcytotoxic stress apoptotic pathways are slightly activated but overcome by growth arrest and SIPS pathways.

ROS have been shown to increase at later cumulative population doublings in HDFs (Atamna *et al.*, 2000 for arguments). Protein oxidation also increases with cumulative population doubling in proliferating BJ HDFs, perhaps as a result of decreased proteasome and lysosomal cathepsin activity. It was suggested that oxidized proteins accumulate throughout life until reaching a level that inhibits the proteasome (Sitte *et al.*, 2000a). Interestingly, similar results were found in confluent BJ HDFs (Sitte *et al.*, 2000b). Yet if RS in BJ HDFs derives from the telomeres, the protein oxidation should be a result, not consequence of these processes. We suggest such studies should be extended to immortal hTERT-BJ1 HDFs to assess the causal relationship between protein oxidation and RS. Unpublished data from our laboratory, however, suggests that proteasome activity is not impaired in H_2O_2-, UVB-, and t-BHP-induced SIPS in WI-38 and AG04431 HDFs. Moreover, the data on decreased proteasome activity (Sitte *et al.*, 2000a & 2000b) shows that the decreased proteasome activity occurs after many weeks of hyperoxia at 40% O_2, suggesting that the cells were not only in SIPS but also ready to die. A decrease in proteasome activity was observed in our laboratory at several days after highly cytotoxic H_2O_2 stress (unpublished).

As mentioned in chapter 3.2.1., other proteins, such as ATM, ATR, and DNA-PK may be responsible for sensing DNA damage (Yang *et al.*, 2003 for arguments). Although ATM has been hypothesized as a sensor of stress (Rotman and Shiloh, 1997), recent results suggest that the ATM-dependent pathway is not critical for SIPS induced by oxidative stress, at least in some cell lines (Naka *et al.*, in press). Also recently, it has been shown that the early growth response 1 protein acts upstream of p53 in mouse cells (Krones-Herzig *et al.*, 2003). On the other hand, recent results show that CDK4 overexpression upregulates $p16^{INK4a}$, p53, and $p21^{WAF1}$ and yet bypasses the $p16^{INK4a}$-associated senescence due to telomere-independent stress (e.g. inadequate culture conditions) and telomere-dependent senescence (Ramirez *et al.*, 2003). Therefore, our work is but a small glimpse of the complex mechanisms involved and much work remains before we fully understand the cell cycle and its regulatory loops. Even so, we think SIPS is caused by the DNA-damage response deriving from genotoxic damage.

10.3. RS versus SIPS

One discussion is whether RS and SIPS are the same process. As mentioned in chapter 2.4., there are clear similarities and differences between the two. At a proteomic level, the

protein expression changes observed in RS were only partially observed in SIPS (Dierick *et al.*, 2002). Our results also suggest differences in gene expression between RS and SIPS. It has been proposed that changes specific of SIPS and/or changes specific of each model of SIPS are "molecular scars" of subcytotoxic stresses (Brack *et al.*, 2000). Therefore, one hypothesis is that RS and SIPS are different processes, as suggested by others (Wright and Shay, 2001).

Although telomere dysfunction is the main cause of RS, considerable gene expression differences have been verified in different cell lines during RS. The position of the co-expressed genes along the chromosomes appears clustered; a phenomenon termed chromosomal clustering (Zhang *et al.*, 2003). Due to the stochastic nature of ageing, even if RS originates in a common mechanism, the phenotypic changes may differ. As such, another possibility is that RS and SIPS are the same process despite some phenotypic differences.

Another way of comparing RS and SIPS is by comparing the underlying mechanisms. If we consider RS as either derived from telomere signalling cascades or inadequate culture conditions, then even though the role of telomeres in SIPS may be less prominent than in RS, SIPS and RS are the same process, each capable of originating from two or more signalling cascades. Besides, the role of pRb and p53 in RS and SIPS proves that there are common players involved, suggesting, for instance, that both RS and SIPS evolved as anti-tumour mechanisms. Others have suggested that telomere-independent mechanisms of cellular senescence should not be considered RS (Wright and Shay, 2001). If we follow this definition then RS and SIPS are clearly not the same process. The question thus is how RS should be defined. We think the debate of RS versus SIPS is a futile one because what matters is the understanding of the mechanisms involved and how they relate to human biology. Whether telomere-induced senescence should be named RS and telomere-independent senescence should be named something else is irrelevant from the perspective of understanding ageing and is a discussion that will generate more heat than light. Lastly, it is important to find which mechanism is predominant *in vivo*. For example, in rat kidneys, $p16^{INK4a}$ levels increase dramatically with growth and ageing. SA β-gal was also associated with ageing in the epithelium. Yet these changes occurred without significant telomere shortening (Melk *et al.*, 2003), so telomere-independent mechanisms prevail, at least in some stress-prone tissues as mentioned in chapter 2.4.

10.4. DNA metabolism and ageing

Despite the large number of theories of ageing, only a fraction of genes has been identified that is capable of modulating ageing in mammals. Many genes that affect ageing in mammals are involved in DNA metabolism. For example, mice with a mutation in *XPD*, a gene encoding a DNA helicase that functions in both repair and transcription, feature a phenotype resembling accelerated ageing (de Boer *et al.*, 2002). RS in human cells derives essentially from changes in the DNA, namely, in the telomeres (Bodnar *et al.*, 1998). Even SIPS in our model involving BJ cells is likely to result from the genotoxic action of H_2O_2. In addition, pRb interacts with lamin A/C (Mancini *et al.*, 1994), and recent results suggest that pRb mediates heterochromatin formation during RS (Morrison *et al.*, 2002; Narita *et al.*, 2003). Histone deacetylase antagonizes p53 (Lagger *et al.*, 2003) and chromatin structural changes are involved in RS (Ning *et al.*, 2003; Zhang *et al.*, 2003), though how this correlates with human ageing remains undetermined. Therefore, it appears likely that the DNA plays an important role in ageing (de Magalhaes *et al.*, in press). As mentioned in chapter 1.3.3., the idea that functional or structural changes in chromosomes are at the basis of ageing is not new. To quote Leonard Guarente (Guarente, 1996): "It would perhaps be appropriate that chromosomes, which orchestrate the genesis, development, and maturation of organisms, also direct the final chapter in the life cycle."

Mice deficient for topoisomerase IIIβ develop normally and yet have a reduced longevity (Kwan and Wang, 2001). Though it is only an evolutionary distant model, mutations in the yeast Sgs1 protein--with homology to WRN and other helicases--cause accelerated ageing in yeast (Sinclair *et al.*, 1997). Interestingly, Sgs1 interacts with topoisomerase III (Ng *et al.*, 1999; Duno *et al.*, 2000). WS cell lines have been previously shown to be hypersensitive to topoisomerase inhibitors (Pichierri *et al.*, 2000a & 2000b). Although the exact mechanisms are unclear, these results suggest a role for DNA structure in WS.

Many results support the role of WRN in DNA metabolism (reviewed in Opresko *et al.*, 2003): WRN interacts with p53 (Blander *et al.*, 1999) and can induce p53 in response to DNA damage (Blander *et al.*, 2000); interestingly, p53-mediated apoptosis is attenuated in WS fibroblasts (Spillare *et al.*, 1999). The ability of WRN to attack unusual DNA formations (Orren *et al.*, 2002 for arguments) suggests that WRN functions as a key factor in resolving aberrant DNA structures to maintain the genetic integrity in cells (reviewed in Shen and Loeb, 2000; Bohr *et al.*, 2002; Fry, 2002). Recent results also implicate chromatin alterations in the pathology of WS (Kyng *et al.*, 2003).

The interaction between WRN and the telomeres is also intriguing. Mean telomere loss is accelerated in WS fibroblasts but these exit the cell cycle with longer telomeres than normal, senescent human cells (Schulz *et al.*, 1996). Intriguingly, TRF2 binds and activates WRN (Opresko *et al.*, 2002). WRN interacts with Ku, suggesting that WRN is involved in telomere maintenance (Li and Comai, 2000), as happens with Sgs1 (Johnson *et al.*, 2001). hTERT expression in WS cells prevents RS but does not fully restore WS cells into normal cells (Choi *et al.*, 2001). Interestingly, recent results indicate that WRN may downregulate telomerase (Bai and Murnane, 2003). Either telomere imbalance plays a role in the phenotype of WS or rather the accelerated telomere shortening is a sign of genomic imbalance and a consequence of WS. The recent findings showing an inverse correlation between telomere shortening and bird and mammalian longevity support this hypothesis (Haussmann *et al.*, 2003; Vleck *et al.*, 2003), as do some preliminary results showing that at least in some turtles' telomere shortening does not occur *in vivo* (Girondot and Garcia, 1999).

Our proposal is that chromatin changes accumulate with ageing making cells respond differently to insults and stress. Changes in chromatin remodelling genes make yeast more susceptible to stress (Tsukiyama *et al.*, 1999). Other results from yeast also suggest that chromatin structure may play a role in yeast ageing (Lin *et al.*, 2000), as proposed by others (Guarente, 1996, 1997; Campisi, 2000; Bitterman *et al.*, 2003). Although limited, some data on mammals also suggest chromatin changes during ageing. Heterochromatinization may change during human ageing (Lezhava, 2001). Results from mice also suggest age-related chromatin changes during ageing (Lindner *et al.*, 1999; Sarg *et al.*, 2002). Of course that these changes may be a result rather than a cause of ageing. Chromatin condensation is a signal of apoptosis (Arends *et al.*, 1990; Collins *et al.*, 1994). If the number of apoptotic cells increases with age it is normal to find a correlation between chromatin condensation and age. Yet the involvement of WRN in ageing and DNA metabolism, and to a less extend of lamin A/C (Glass *et al.*, 1993; Gotzmann *et al.*, 1999; Stierle *et al.*, 2003), the way WRN cells are more sensitive to oxidative stress, and the way chromatin structural changes seem to occur faster in WS cells (Almagor and Cole, 1989) lead us to argue that chromatin structural changes are a key regulating factor in human ageing (de Magalhaes *et al.*, in press).

CHAPTER 11: HOW BIOINFORMATICS CAN HELP REVERSE ENGINEER HUMAN AGEING

One of the problems in gerontology is that there is much data but few explanations and no criteria on how to assimilate the most important information. Since mathematics and statistics give a meaning to data, we wanted to develop ways to turn data into knowledge. Through bioinformatics, researchers can gather and store large amounts of data and use computing power to develop more complex models (reviewed in Baldi and Brunak, 2001). The integration of information from several sources is problematic, particularly in ageing with conflicting results from different models. In truth, a combination of both computer-based and experimental approaches will be necessary to understand a complex process such as ageing. Consequently, we wanted to develop strategies on how to integrate data from different models into one coherent model of human ageing. We propose a systems biology strategy for modelling human ageing by integrating data from different models. This part of the discussion is in press at *Ageing Research Reviews* (de Magalhaes and Toussaint, in press).

11.1. Introduction

Ageing is an intrinsic age-related process of loss of viability and increase in vulnerability (Comfort, 1964). Studying human ageing has two major difficulties: the complexity of the ageing phenotype with its widespread changes and pathologies associated with chronological age and the near impossibility of performing *in vivo* studies. Consequently, most researchers resort to models that may or may not be accurate representations of the human ageing process. So to understand human ageing is an enormous challenge, not only due to the complexity inherent to ageing but also since our hypotheses are based on extrapolations and our theories will have to be tested indirectly.

It is not surprising then that human ageing remains a mysterious process. We still cannot answer the most important questions: Why do species age at different paces? What determines the rate of ageing? How can we distinguish causes from effects of ageing? What changes occur in an adult human being to make the chances of dying duplicate approximately every eight years? In the end, why do we age? So far we already know several genes that modulate rate of ageing in animals, such as $p66^{shc}$ (Migliaccio *et al.*, 1999), but, optimistically, only a few in humans such as the progeroid genes responsible for Werner's (Yu *et al.*, 1996) and Hutchinson-Gilford's syndromes (Eriksson *et al.*, 2003). Yet these genes alone show the extraordinary

influence of genetic mechanisms on ageing. Just discovering more genetic players in the human ageing process would be a major breakthrough.

Scientific discovery has always been limited by the available technologies. Given the modern methods to gather huge amounts of data, such as the recent genome sequencing efforts (Lander *et al.*, 2001; Venter *et al.*, 2001; Waterston *et al.*, 2002), computational methods offer a powerful set of tools to help understand the ageing process and its genetic network (reviewed in Luscombe *et al.*, 2001; Yaspo, 2001). In this article, our objective is to focus on computational tools that may be useful for the study of human ageing by: (1) data-mining methods, such as comparative genomics, phylogenetic footprinting, and DNA microarrays, that may be employed to gather information about ageing; (2) *in silico* methodologies aimed at interpreting information, such as algorithms to understand complex networks, that may be useful to model the genetic network of human ageing.

11.2. Data-mining methods

Computational data-mining approaches are particularly appropriate in areas with much data but few explanations, such as gerontology. If researchers can find patterns in data to perceive information, then information may enhance our knowledge over ageing. The goal of applying computational data-mining approaches is to extract useful information from large amounts of data by employing mathematical methods that should be as automated as possible.

11.2.1. Comparative genomics of ageing

Having the human genome offers the digital genetic code that is the source of ageing while having several fully sequenced genomes offers data-mining opportunities to decipher that code. Since genomes had a common ancestor, every base pair in each organism can be explained as a combination of the ancestral genome with evolution, and so comparing different genomes is a powerful way to analyze and interpret genome sequence. Comparative genomics allows researchers to, for instance, discover new genes, assign function to unknown genes, and gather information on protein interactions (reviewed in Wei *et al.*, 2002; Ureta-Vidal *et al.*, 2003).

Early studies using the several available microbial genomes already showed how comparative genomics can be used to gather information about gene function (reviewed in Yaspo, 2001; Wei *et al.*, 2002). One method involves determining the presence or absence of proteins across several genomes to build a phylogenetic profile for each one. Functionally related

proteins are expected to have similar profiles and thus can be grouped together, giving hints on the function of several unknown proteins (Pellegrini *et al.*, 1999). Another approach to find functionally related proteins involves a "Rosetta stone" analogy. In brief, if the homologues of a pair of proteins in an organism are found fused into a single protein chain in another organism's genome, it can be used to infer a functional interaction (Marcotte *et al.*, 1999a). Lastly, more detailed comparisons may be performed. In one example, the DNA repair proteins of *Escherichia coli* and *Saccaromyces cerevisiae* were compared to the entire sets of protein sequences of several fully sequenced genomes. Multiple alignments of the protein families found were constructed using algorithms such as ClustalW (Thompson, 1994). This method allowed an identification of novel enzymatic and DNA-binding domains involved in repair, as well as an improved view on the evolution of repair systems and how these depend on environmental conditions and the cell's physiology. By identifying domains with disrupted functional motifs, researchers can also predict proteins without enzymatic activity (Aravind *et al.*, 1999).

It may be possible to discover novel proteins involved in ageing through phylogenetic profiles using as reference, for example, proteins involved in progeroid syndromes. In addition, many different organisms exhibit extraordinary similar ageing phenotypes at radically different paces. For example, some primates such as baboons (Bronikowski *et al.*, 2002) or rhesus monkeys exhibit a similar ageing process to humans but twice as fast and mice age 25-30 times faster than humans do (Finch, 1990); on the other hand, whales appear to age slower than humans (George *et al.*, 1999). These observations are independent of environmental conditions, suggesting that genetic factors are largely responsible for rate of ageing in mammals (Miller, 1999; de Magalhaes, 2003). Although different rates of ageing may also be due to metabolic rates, these alone do not explain the variety in mammalian rates of ageing (Austad, 1997). Eventually, it will be possible to compare whole sets of protein families across mammalian species with different rates of ageing in hope of finding some sort of correlation, for example, in the form of expanded or contracted protein families.

Since the digital core of information from which ageing arises is ultimately knowable (Hood, 2003), comparative genomics offers an accessible set of tools to study ageing in humans. The major limitation in applying the described methodologies to mammals is the need for several fully sequenced genomes, which should occur within a reasonable future. For example, expansion and contraction of protein families in mice--mostly reproduction, immunity, and olfaction--do not suggest any involvement with ageing (Waterston *et al.*, 2002). Yet with the prospect of having several fully sequenced mammalian genomes within the next few years--namely, human, mouse, rat, chimpanzee, rhesus macaque, cow, pig, and dog genomes

(http://www.genome.gov/page.cfm?pageID=10002154)--comparative genomics offers a powerful approach to study human ageing.

11.2.2. Transcriptional regulation of ageing

One of the major discoveries of the sequencing of the mouse and human genomes is the extraordinary genetic similarity between mice and humans. According to the latest projections, mice share roughly 99% of the total number of human genes (Waterston *et al.*, 2002), and some scientists argue that humans and their close relatives--e.g. chimpanzees--have the same set of genes (for example: Wade, 2001). Recent evidence indicates that alterations in transcription discriminate humans from other primates, not different genes (Enard *et al.*, 2002). It appears that the genes may well be very similar in humans and other animals, but they are used or transcribed differently and the way they are transcribed determines the differences between humans and other animals (Hood and Galas, 2003; Levine and Tjian, 2003). Therefore, transcriptional regulation may be a key in mammalian ageing, as already suggested (Roy *et al.*, 2002; Hood, 2003).

Transcriptional regulation is a complex process that only recently began to be understood (reviewed in Arnone and Davidson, 1997; Fickett and Wasserman, 2000). Importantly, transcriptional regulation is also digital in nature. It is located in the non-coding genetic sequence, largely in the form of *cis*-regulatory sequences that are critical in development and cellular differentiation. These regulatory sequences are specific targets of transcription factors (TFs) that, together with proteins that bind them, control gene activity. Since transcriptional regulation is located in the genetic information, bioinformatics is also attempting to tackle regulatory networks.

One promising computational tool for the identification and characterization of regulatory regions is phylogenetic footprinting (reviewed in Hardison *et al.*, 1997; Pennacchio and Rubin, 2001; Ureta-Vidal *et al.*, 2003). As with comparative genomics, the principle behind phylogenetic footprinting is that regulatory regions tend to be conserved across different species. One example of how phylogenetic footprinting can be used involves the stem cell leukaemia (*SCL*) gene. Briefly, clones containing the human, mouse, and chicken *SCL* gene were sequenced. Comparing human and mouse sequences revealed several homology peaks, a subset of which corresponded to previously known *SCL* enhancers. Chicken sequences corresponding to human/mouse peaks identified a conserved non-coding region with no known functions. Using a transgenic reporter assay, it was possible to characterize that region as a new neural

transcriptional enhancer (Gottgens *et al.*, 2000). In other experiments, phylogenetic footprinting has been used in the discovery of mammalian regulatory elements of genes such as Bruton's tyrosine kinase, β-globin (Hardison *et al.*, 1997), and interleukins 4, 5, and 13 (Loots *et al.*, 2000). With the availability of more genomes, phylogenetic footprinting should gradually become more powerful (for example: Hardison *et al.*, 1997).

Since TFs bind specific sequences, it is possible to find putative transcription factor binding sites (TFBS) in genome sequence through computational algorithms (reviewed in Stormo, 2000; Ohler and Niemann, 2001; Pennacchio and Rubin, 2001). One major problem with putative TFBS identified by computational methods, and, to a lesser degree, in phylogenetic footprinting, is the presence of false positives. Experimental verification is often necessary. In addition, TFs in metazoans often interact with each others, making it difficult to find the complete set of regulatory interactions. To minimize this problem, more efficient algorithms, or combination of algorithms, can search clusters of TFBS or TFBS conserved between mouse and human sequence (Wagner, 1999; Levy and Hannenhalli, 2002). Although these methods are becoming increasingly efficient, the true power of putative TFBS detection emerges when taken together with other types of data, as will become apparent ahead.

The principle in studying transcriptional regulation may also involve a comparative biology approach in an attempt to understand why different mammals age at different rates. Only instead of searching genes, we would be searching regulatory sequences. The importance of transcriptional regulation should not be underestimated. For example, as much as 40% of human TFBS are not functional in rodents (Dermitzakis and Clark, 2002). Using phylogenetic footprinting it may be possible to find the TFs that control key genes involved in ageing while detecting putative TFBS may help us gather hints about mechanisms of ageing. If indeed the differences in rate of ageing are due to subtle transcriptional differences amongst mammals, then the study of transcriptional proteins and regulatory sequences will be relevant for ageing research. In other words, if ageing and cancer can be seen as the corruption of the genetic program, then the study of transcriptional regulation might allow researchers to understand why it becomes corrupted with age.

11.2.3. DNA microarrays

DNA microarrays based on the quantification of mRNA levels are a growing technology (reviewed in Lockhart and Winzeler, 2000). Although the correlation between mRNA and protein levels is not always linear (Gygi *et al.*, 1999), DNA microarrays have the technological

edge over proteomics and protein microarrays (reviewed in Pandey and Mann, 2000; MacBeath, 2002) due to their capacity to produce large amounts of gene expression data from different conditions in relatively little time. The principle is that changes in mRNA levels between different conditions also reflect changes in the system under study and often at a protein level.

Studies of complex processes in yeast have showed the power of DNA microarrays by measuring mRNA levels for practically every yeast gene. One experiment attempted to relate the gene expression program to the sequence of events in sporulation by measuring gene expression at t = 0, 0.5, 2, 5, 6, 7, 9, and 11.5 hours during sporulation. By grouping genes according to their expression profiles during sporulation, it was possible to find functional links and provide clues about the function of previously uncharacterized genes. Clustering of gene expression patterns allowed hints about the function of hundreds of genes and, for example, a 10-fold increase in the number of identified genes that participate at the middle stages of sporulation. DNA microarrays have also become crucial to understand transcriptional regulation. In the same experiment, consensus sequences for the *USR1* transcriptional regulator were found by computational approaches upstream of the start codon of many genes clustered according to expression patterns. These results allowed an association between a temporal pattern of gene expression, a stage in sporulation, and a transcriptional regulator with its respective regulatory sequence. The role of the Ndt80 TF was also investigated through either ectopically expressing Ndt80 or eliminating it. In theory, this approach can be used to find nearly every gene regulated by a TF under the experimental conditions used, though false negatives may exist since genes are often controlled by multiple TFs. Together with analysis of putative Ndt80 binding sites, this work allowed the identification of several genes presumably controlled by Ndt80 (Chu *et al.*, 1998).

Clustering genes with similar expression patters with age may allow researchers to find functional links. Yet the objective in applying DNA microarrays to study ageing is to relate the gene expression program with the sequence of events of the ageing process in hope that will allow us to determine the regulation of ageing. One major problem is that, unlike yeast sporulation, the ageing process may not be programmed. Others have suggested that age-related changes in gene expression are deleterious for they represent a shift from what is assumed to be a young pattern (for example: Lee *et al.*, 1999; Jiang *et al.*, 2001). Yet instead of indicating causes of ageing, gene expression profiles as animals age may represent the tissue's response to ageing. For instance, if ageing derives from damage accumulation, then gene expression patters will change with age as a response to damage. Therefore, due to the unique and unclear basis of ageing, data obtained from gene expression patterns must be carefully interpreted. For example, one study on cellular senescence found disparate changes in gene expression between two

different cell types despite the same telomere-dependent mechanism. Yet up-regulated senescence-specific genes showed chromosomal clustering between both cell types, leading to the suggestion that chromatin changes are involved in cellular senescence (Zhang *et al.*, 2003). It is clear that changes occur in human cells as we with age, but gene expression profiles may only reflect a response to those changes rather than the changes themselves.

One important application of DNA microarrays for the study of ageing would be to find markers of ageing. For example, DNA microarray analysis of malignant lymphomas has proven useful in detecting genes that can be employed to classify and predict the prognosis of tumours (reviewed in Schwaenen *et al.*, 2003). Finding gene expression patterns capable of serving as indicators of how aged an animal or a human is would prove useful for research. For instance, if the recent advances in sequencing power are anything to go by, then in 10 years we may be able to sequence a human genome in one day (for example: Hood, 2003). Having a method to measure biological ageing would allow us to calculate the pace of ageing in individuals and possibly find clues about the genetic players that modulate the ageing process. Even now, such method would allow us to compare, for example, single-nucleotide polymorphisms amongst individuals with different paces of ageing.

Other technical variants and applications of DNA microarrays exist (reviewed in Lockhart and Winzeler, 2000). One powerful technique for the understanding of transcriptional regulation is genome-wide location analysis (reviewed in Wyrick and Young, 2002). In yeast, the technique involves a DNA microarray with the complete set of yeast intergenic regions. DNA enriched with an antibody against the TF of interest is labelled and hybridized against the microarray revealing which promoters, and often which genes, are regulated by the TF. This technique has also been used to study the E2F TF family in human cells using a selection of about 1,200 human promoter sequences. Several genes were identified as potentially activated by E2F, including many genes with no previous connection to E2F (Ren *et al.*, 2002).

Applying genome-wide location analysis to study ageing is a potentially powerful approach not only in understanding cellular senescence but also the ageing process. It could shed light on what genes are regulated by TFs suspect of participating in ageing--for example, the redox-regulated TFs such as AP-1, Sp1, and NF-κB (Lavrovsky *et al.*, 2000). Furthermore, it could help elucidate the roles of these and other TFs at different ages. For instance, understanding which genes are activated at different ages by p53 could help clarify p53's role on ageing and cancer mechanisms. Of course we may face the same problem of finding responses to ageing rather than causes. If genes do not exist to cause ageing, then neither do regulatory

sequences. Yet these respond to stimulus and if these responses change during ageing then finding them may help understand what causes ageing.

When applying DNA microarrays to the study of ageing we face the problem of how to focus on human ageing. It appears difficult to obtain data on gene expression at different ages for humans using longitudinal studies. One idea could be to use cross-sectional studies. Although such studies are obviously biased by the variability amongst individuals (Hofer and Sliwinski, 2001), if done on a sufficient number, cross-sectional studies may prove useful (Weindruch *et al.*, 2002). Of course, cross-sectional studies appear the only solution to study long-lived animals, such as whales or turtles, and they have already been employed to study, for example, rhesus monkeys (Kayo *et al.*, 2001). Therefore, at least using longitudinal studies, we are likely to depend on model organisms.

Studying ageing in animals has the additional problem of having to deal with several different cells types--over 200 in vertebrates (Alberts *et al.*, 1994). As far as we know, ageing can be caused simultaneously in all tissues, it can be a result of changes in a particular organ, or it can even involve different genetic programs at different tissues. Indeed, previous studies already indicated that gene expression profiles are specific for the ageing process of each organ (reviewed in Weindruch *et al.*, 2002). Interpreting gene expression profiles in multicellular organisms is a difficult problem, which will rely on having a substantial amount of data from different tissues to allow researchers to isolate which age-related gene expression changes are passive effects, causes, or responses to ageing. Techniques for measuring tissue-specific gene expression have been applied to *Caenorhabditis elegans* (reviewed in Reinke, 2002) and could be employed in mammals to discriminate tissue-specific age-related gene expression changes. Lastly, different forms of data-analysis, such as chromosomal clustering (Zhang *et al.*, 2003), may reveal information that is otherwise unobvious.

In conclusion, DNA microarrays may provide much data and even information for the study of ageing. The great advantage of DNA microarrays is that it is not necessary to know what genes are important in the process under study. Large amounts of age-related gene expression data from different tissues of, for instance, mice, would prove an invaluable resource for the study of ageing. At present, microarray databases, such as Standford's (Sherlock *et al.*, 2001), already contain a few datasets regarding ageing (reviewed in Jennings and Young, 1999). With time, researchers can hope to have the age-related transcriptome of different tissues from several model organisms.

11.3. Modelling human ageing

Computational methods offer several data-mining tools, as previously mentioned, and an exponentially increasing amount of data (Table 4). To understand biological systems, the amount of data needed is colossal because biological systems are intrinsically complex with diverse, often multifunctional, elements that interact in nonlinear ways (Toussaint *et al.*, 1991). In turn, data and information can be analyzed and interpreted by way of computer and mathematical methods to create models that are easier to study than biological systems. Ultimately, these models allow us to increase our knowledge about the process under study. If we could simulate, for instance, cellular senescence *in silico*, it would be a major development. Yet the grail of gerontology is the reconstruction of the genetic network of human ageing: the identification of the causal structure of the ageing process's gene network.

11.3.1. System structure and identification

Uncovering a complex process such as human ageing will depend on the employment of both computational tools and experimental approaches. The integration of these two forms of information requires a systems biology approach (reviewed in Ideker *et al.*, 2001a; Kitano, 2002a, 2002b). Systems biology is based on information--e.g. the quantification of a gene product--obtained from a given biological system under different genetic and/or environmental conditions. Information is then mathematically treated to construct a model that explains the system. For example, insights into the regulation of galactose use (*GAL*) in yeast have been obtained through systems biology. To study the *GAL* pathway, mRNA and protein data were obtained from yeast strains under different environmental and genetic conditions. Exemplifying, strains were examined each with a different *GAL* gene deleted. Using previously known protein-protein and protein-DNA interactions, previous models of the *GAL* pathway, and the new data, it was possible to build an integrated physical-interaction network for over 300 genes. Several putative interactions were also identified through gene expression analysis combined with TFBS scans or simply by searching genes with correlated expression profiles. New regulatory phenomena were also proposed, some of which later experimentally verified (Ideker *et al.*, 2001b).

Applying systems biology to study human ageing is not straightforward. The accuracy and detail of a model is dependent on how much data we can gather in how many different circumstances (Selinger *et al.*, 2003). From a mathematical perspective, we can imagine

Table 4

List of major databases and bioinformatics websites

Name:	Website:
EMBL	http://www.embl-heidelberg.de/
EMBL's genome browser	http://www.ensembl.org/
European Bioinformatics Institute	http://www.ebi.ac.uk/
NCBI	http://www.ncbi.nlm.nih.gov/
TIGR	http://www.tigr.org
USCS Genome Bioinformatics	http://genome.ucsc.edu/

Selection of databases that may be useful to gerontologists

Baltimore Longitudinal Study of Aging	http://blswww.grc.nia.nih.gov/
BodyMap	http://bodymap.ims.u-tokyo.ac.jp/
GeneCards	http://bioinformatics.weizmann.ac.il/cards/
Human Protein Reference Database	http://www.hprd.org
Protein Data Bank	http://www.rcsb.org/pdb/
SAGE KE database of genes/interventions	http://sageke.sciencemag.org/cgi/genesdb
Swiss-Prot	http://us.expasy.org/sprot/
Telomere Database	http://www.genlink.wustl.edu/teldb/index.html

modelling ageing as mapping the rules that make a young organism old. If a large number of genes are involved in the process, as it appears likely, then a large number of measurements are necessary to understand the rules governing those genes. The duration of ageing even in animal models makes ageing a difficult subject of study when compared to, for instance, yeast sporulation. In addition, a major limitation to study ageing in humans or model organisms, such as mice, is the relatively small amount of ways to change ageing (Table 5). Therefore, and since our understanding of the ageing process is still in its infancy, modelling human ageing will have to be accomplished in phases. The first phase must be the identification of the elements involved in the human ageing process, possibly with knowledge about interactions between them. In addition, we must attempt to learn more about the structure of the ageing process. For instance, does ageing derive from damage accumulation, programmed gene expression changes, changes in DNA structure, or some other process?

Experimental approaches have already proven useful in identifying a few genes that may be involved in human ageing. The new computational tools described earlier will be determinant to find novel genes involved in ageing. DNA microarrays, in particular, are a powerful approach. For example, studying cellular processes such as stress response and DNA repair may help us gather clues about functional interactions between proteins suspect of being involved in ageing (Table 5) and previously uncharacterized proteins. If indeed certain progeroid syndromes in both mice and men are cases of accelerated ageing, then finding new functional links involving these proteins is a promising approach. Also, large-scale gene expression profiles of ageing animals may help clarify the structure of the ageing process, something that so far eluded researchers.

11.3.2. System-level perturbations in model organisms

As we identify the elements of the ageing process we can attempt to predict its progress by perturbing each component of the system. Due to the duration of human ageing, model organisms will play a critical role. Namely, perturbations of ageing in model organisms by, for instance, genetic interventions will be crucial to obtain the amount of data necessary to understand ageing. Another type of perturbation results from evolution and the way several species have different rates of ageing.

The major problem of using animal models is that the genetics of ageing in model organisms may or may not be similar to the genetics of ageing in humans. For example, mutations in the mouse homologue to the Werner's syndrome gene have no visible effect on their ageing process (Lombard *et al.*, 2000; Wang *et al.*, 2000). One critical paradox in studying

Table 5

Major perturbations of the aging phenotype in mice

Name	Perturbation	Reference
Ames dwarf	Homozygous mice show over 50% increases in life-span.	Brown-Borg *et al.*, 1996
Caloric restriction	Delay of the aging process.	Weindruch and Walford, 1998
GHR (growth hormone receptor)	Increase in life-span of 40-50% in homozygous knock-outs.	Coschigano *et al.*, 2000
Ghrhr (Growth hormone releasing hormone receptor)	Life-span increase of about 20% in homozygous knock-out mice.	Flurkey *et al.*, 2001
IGF-1R (insulin-like growth factor receptor)	Heterozygous mice live 26% longer than wild-type.	Holzenberger *et al.*, 2003
klotho	Possible accelerated aging phenotype of homozygous knock-outs.	Kuro-o *et al.*, 1997
p53	Heterozygous mutant mice display signs of accelerated aging.	Tyner *et al.*, 2002
$p66^{shc}$	Roughly 30% increase in life-span in -/- mice.	Migliaccio *et al.*, 1999
Snell dwarf mice	Life-span increase of 42% in homozygous mice.	Flurkey *et al.*, 2001
Telomere dysfunction and ATM deficiency	Possible accelerated aging in double mutant mice.	Wong *et al.*, 2003
urokinase-type plasminogen activator	Roughly 20% increase in life-span of transgenic mice.	Miskin and Masos, 1997
XPD (xeroderma pigmentosum, group D)	Possible accelerated aging phenotype due to homozygous mutation.	de Boer *et al.*, 2002

Potential perturbations of the aging phenotype in humans

Name	Perturbation	Reference
CKN1 (Cockayne Syndrome Type I)	Possible accelerated aging due to mutation.	Henning *et al.*, 1995
WRN (Werner Syndrome gene)	Premature aging due to recessive mutation.	Yu *et al.*, 1996
LMNA (lamin A)	Possible premature aging due to dominant mutation.	Eriksson *et al.*, 2003

ageing is that as we move across evolution in search of models closer to humans, these have increasingly longer life spans, making them increasingly more difficult and expensive to study. For example, studying genetic perturbations is much easier in *C. elegans* than in mice, but genes found in mice have more chances of being successfully extrapolated to humans than genes found in *C. elegans*. Although studies in, for instance, invertebrates will continue to yield putative genes involved in human ageing (for example: Tower, 2000; Hekimi and Guarente, 2003), we must have a coherent view of ageing mechanisms in several species, including mammals, before we can extrapolate conclusions to human ageing.

Model organisms have already given useful insights on biological complexity and on the organization and dynamics of the ageing process. Some successful work using DNA microarrays has been done in *Drosophila melanogaster* (for example: Zou *et al.*, 2000), *C. elegans* (for example: Lund *et al.*, 2002), and mice (for example: Lee *et al.*, 1999, Cao *et al.*, 2001; Jiang *et al.*, 2001; Miller *et al.*, 2002). Interestingly, some of the studies employing DNA microarrays on mice also studied caloric restriction and/or life-extending mutations (for example: Lee *et al.*, 1999; Cao *et al.*, 2001; Miller *et al.*, 2002). If the mechanisms of ageing are similar amongst mammals, as it may be the case (de Magalhaes and Toussaint, 2002), then mammalian ageing is a combination of the basic mechanisms of ageing, metabolic rates, and species-specific traits. Gene expression studies comparing different primates as well as different rodents have already been used to identify species-specific patterns of expression (Enard *et al.*, 2002). Performing such studies using a temporal resolution of ageing, at least in rodents, would allow us to determine the progression of gene expression as animals age, thus helping us find common responses to ageing or even causative factors in mammalian ageing.

If ageing and development are two independent processes (for example: Miller, 1999), it is also important to identify the changes in animals before vulnerability starts to increase. In other words, to identify what changes occur prior to the sexual peak that make ageing commence. If we aim to develop ways to stop ageing, then this is the question we must ask, not what changes drive senescence in adulthood. Therefore, gene expression changes prior to the increase of age-related vulnerability may reveal important clues about the ageing process, though care must be taken to discriminate such changes from developmental changes.

Although we focus on mRNA quantification and genomics, other data can be gathered such as protein studies (for example: MacBeath, 2002), metabolic fluxes (for example: Nielsen, 1998; Strohman, 2002), and even the study of age-related changes or clinical features of ageing (for example: Mitnitski *et al.*, 2002). The aim is to obtain as much data as possible under as many different conditions to help explain the observed differences. For example, metabolic

control analysis may be applied to study metabolic fluxes in animals under caloric restriction and *ad libitum*-fed controls. Studying what metabolites differ between controls and caloric restricted mice may contribute to our understanding of ageing.

11.3.3. Reconstructing the genetic network of human ageing

Eventually, we would like to create models that allow us to control the ageing process. Due to the limitations on gathering data about ageing, great accuracy in describing human ageing will be impossible in the near future. Therefore, we propose Boolean networks to define the genetic network of human ageing. Simple mathematical descriptions such as Boolean networks classify gene interactions as 1 or 0 (Kauffman, 1993). Given the large amounts of genes presumably involved in a complex process such as ageing, Boolean networks offer a simple and accessible way to model the genetic network of ageing. Although they fail to take into account genes at intermediary levels and are sometimes seen by biologists as inaccurate, Boolean networks make it easy to model complex networks from large amounts of data (Figure 28). Given the complexity of the ageing phenotype, Boolean networks appear as a realistic way to simulate the genetic network of ageing within a reasonable future. A Boolean model of human ageing would already be incredibly useful for medicine in predicting potential anti-ageing interventions.

Several algorithms have been proposed to reconstruct the genetic network of complex processes based on DNA microarray data (for example: D'Haeseleer *et al.*, 2000; Whade and Hertz 2000; Wagner, 2001; de la Fuente *et al.*, 2002; Yeung *et al.*, 2002). Unfortunately, these statistical approaches require amounts of data unrealistic for the present status of ageing research. In addition, DNA microarray data will probably be obtained from model organisms, not humans. Therefore, the greatest challenge in reconstructing the genetic network of ageing will be to integrate data coming from different sources--e.g. DNA microarrays and mutagenesis experiments in model organisms, cellular senescence, and genomics--into one coherent framework, as suggested by others (Jazwinski, 2002; Kirkwood *et al.*, 2003; Hood, 2003).

As mentioned, gene expression data must be combined with other sorts of information--e.g. TFBS prediction--in order for us to understand a system. For example, in one experiment, regulatory motif pairs were used together with gene expression data to study the synergism between them. This approach allowed the identification of novel motif combinations regulating transcription and the modelling of regulatory networks in yeast (Pilpel *et al.*, 2001). If indeed transcriptional regulation plays a key role in ageing, then both data-mining strategies to find the

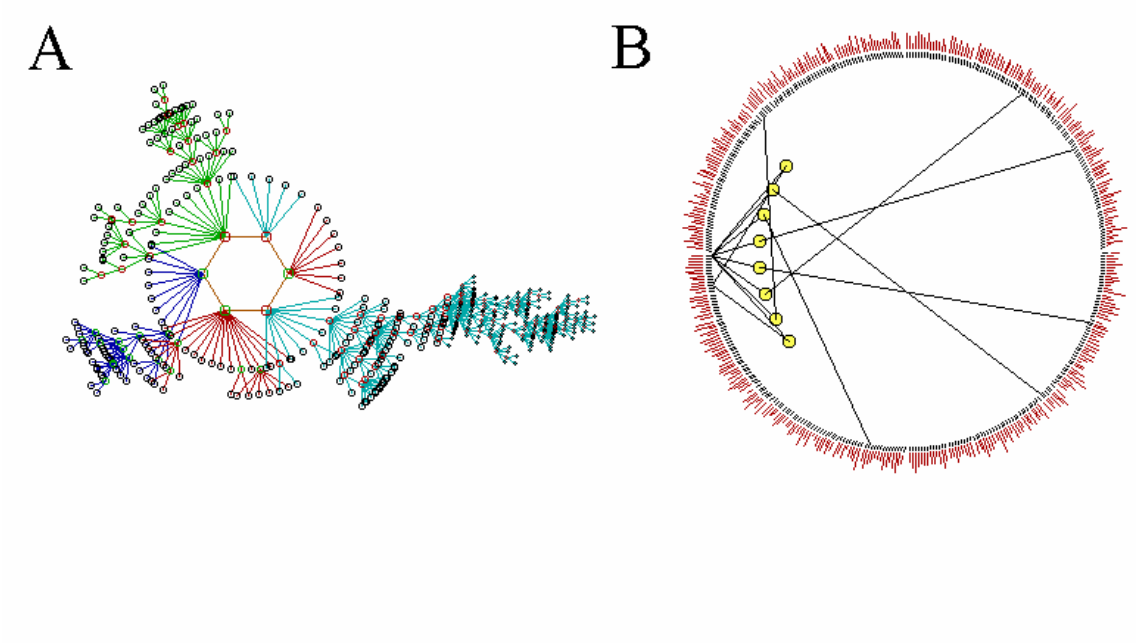


Figure 28: (A) Basin of attraction of a random Boolean network with six attractors. The attractors (centre) are the outcome of the system--for instance, an attractor can be a step in cellular differentiation, a phenotype, even a disease--while the wiring represents biological connections with their own rules describing the level of interaction. Each state can represent, for example, gene activity. Particularly useful for modelling cellular or even yeast cell cycles, Boolean networks also take into account the robustness of biological systems where small perturbations to one state may not affect the general outcome, which is in accordance to what we know about aging--for instance, many diseases accelerate mortality without affecting rate of aging. (B) Cellular automata, or cells, are computer simulations that try to model Nature and life by employing a single set of rules. This image shows the network wiring between two time-steps for a large number of cells (n = 400, on the perimeter), highlighting just one cell. Although these methods are somewhat rough ways of portraying life, they can be much useful in understanding the dynamics and organization of complex genetic networks such as aging. Images generated using DDLAB by Andy Wuensche: http://www.santafe.edu/~wuensch/

relevant transcriptional information and the integration of DNA microarray data from model organisms, such as mice, are crucial. In another example of data integration, researchers combined protein information from experimental data, phylogenetic profiles, correlated mRNA expression levels, and patterns of domain fusions--involving the "Rosetta stone" analogy method--for practically all *S. cerevisiae* proteins. The result was the discovery of 93,000 functional links between proteins which allowed them to assign function to a previously uncharacterized protein family, a yeast homologue of human colon-cancer genes, and the yeast prion Sup35 (Marcotte *et al.*, 1999b). In the end, modelling human ageing will require multiple approaches with several feedback loops. To reconstruct the genetic network of human ageing we need a strategy integrating the discovery of the genes involved with systematic perturbations of ageing (Figure 29).

11.4. Conclusion: is it possible to reverse engineer human ageing?

Reverse engineering is "the process of analyzing a subject system to identify the system's components and their interrelationships and create representations of the system in another form or at a higher level of abstraction." (Chikofsky and Cross II, 1990). To reverse engineer human ageing would be to reconstruct the genetic network of ageing; to find the mechanisms by which a human becomes old and find how to delay and perhaps even reverse the ageing process. In practice, reverse engineer of ageing would allow us to predict which genes actively regulate rate of ageing and eventually what genes could we target to delay human ageing and postpone age-related pathology and degeneration.

In theory, it is possible to reverse engineer a complex process (D'Haeseleer *et al.*, 2000). Yet given the, previously mentioned, limitations in studying human ageing, we find it unlikely that the genetic network of ageing will be understood in detail within a near future. The number of experiments required to fully understand a complex phenotype such as ageing is at present beyond our technology (Wagner, 2001; Krupa, 2002). Yet just finding a few more genes involved in ageing would be a major breakthrough; as it would be to understand the structure of the ageing process. Indeed, others have claimed long ago how the goal of gerontology should be to discriminate causes from effects of ageing and find the one or few physiological processes that control ageing (Medawar, 1955). Although ageing is a complex process that involves many genes and pathways, different genes influence ageing, directly or indirectly, in different ways. In fact, the recent history of the analysis of complex processes shows that even in the most complex of processes we are likely to find key controlling nodes (for example: Risch, 2000; Kitano,

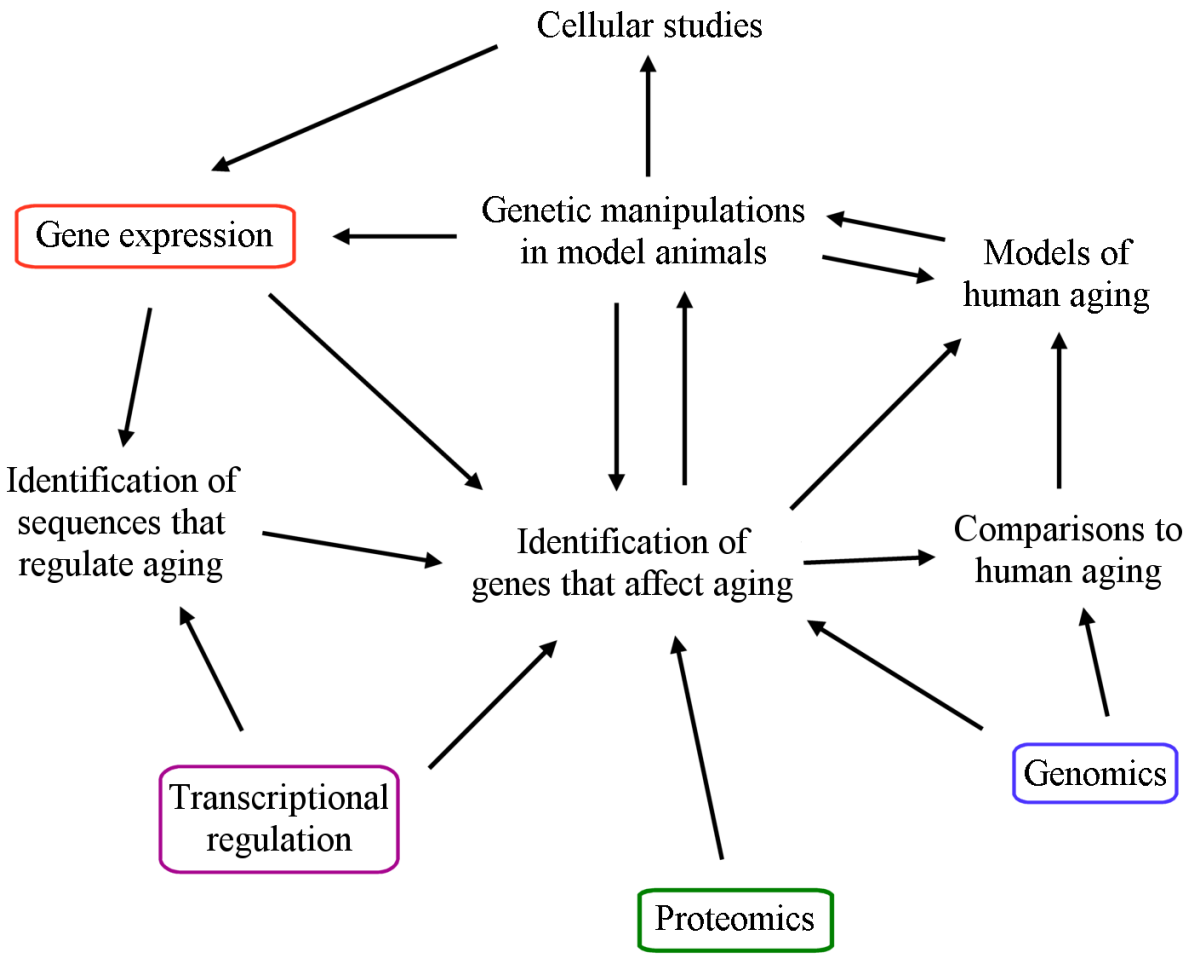


Figure 29: Understanding the genetic network of human aging is based on two general phases: 1) identifying the genetic players involved; 2) systematically perturbing aging and elements/pathways suspect of being involved in aging of model organisms by, for instance, genetic interventions (reviewed in Case, 2003); the integration of results allows us to continually improve our models. A variety of experimental and computational approaches can be used for both aims. Through genetic manipulations, gene expression studies can help discover novel genes involved in aging as well as clarify the role of genes under study. Databases too can be used by, for instance, comparative genomics, to find potential genetic players affecting aging. Comparative genomics can also be used to assess if genes found in model organisms may play a role in human aging. Cellular studies can be useful, for instance, in conjunction with animal models to investigate specific cellular processes. Lastly, studies on transcriptional regulation may be employed to investigate the regulatory signals affecting the genes in question as well as find new elements involved in aging. Colour indicates large-scale databases. Arrows indicate the flow of information.

2002b). Both in practice (for example: Migliaccio *et al.*, 1999) and in theory (for example: Wagner, 2001), it appears possible to locate such nodes in human ageing. Even if we cannot reconstruct the entire genetic network of ageing, locating a few key regulatory processes would be invaluable. For example, if we could develop a draft Boolean model of what genes determine rate of ageing in primates, it would allow us a better understanding of human ageing and possibly the identification of potential therapeutic targets; optimistically, such findings would open the path for the development of ways to delay age-related pathology and senescence.

New tools provide new goals. Until recently, it was unthinkable to attempt to identify all genes involved in ageing. Yet the modern high-throughput technologies allow us to consider the possibility of defining most if not all players involved in ageing as well as how they interact to form the ageing process. Eventually, we will be able to predict the effect of each gene on the ageing process.

General Conclusion

In this work, we developed a refined model of the evolution of human ageing. Our model offers a much more accurate view than the flawed classical evolutionary theory of ageing and contradicts the disposable soma theory. Others too have recently argued in favour of more detailed models to explain the evolution of ageing in mammals (Lee, 2003). The model we developed explains several observations such as why most reptiles feature life-extending traits absent from all studied mammals. In addition, our model led us to conclude that model organisms are not accurate representations of human ageing. If human models are not possible, then mammalian models should be favoured. Although lower life forms can serve as basis for research, we must have a coherent view of mammalian ageing before we can claim we understand the human ageing process. We think that extrapolating findings in lower life forms into human ageing (for example: Howitz *et al.*, 2003) is incorrect. Our work also led us to conclude that studies in long-lived animals such as some turtles and whales may allow us to enhance our understanding over mechanisms of ageing and even lead to the development of anti-ageing interventions. Recent findings support our view (Girondot and Garcia, 1999; Lutz *et al.*, 2003; Christiansen *et al.*, unpublished).

We established a model for the study of SIPS in BJ and hTERT-BJ1 HDFs. Based on our research, the global model of SIPS was improved. We demonstrated that the TGF-β1 pathway does not operate in all cell lines and we were the first to publish SIPS in telomerase-immortalized normal HDFs. Others later confirmed our results (Gorbunova *et al.*, 2002; Matuoka and Chen, 2002). We found no evidence that damage specific to the telomeres is at the origin of SIPS and so our results indicate that the telomeres do not play such a crucial role in SIPS as previously proposed. In particular, telomerase expression does not appear to affect SIPS. Contrary to results at the protein level, we detected an increase in the DNA-binding activity of p53 at 72 hrs after a single H_2O_2 stress. Though in accordance with previously established posttranslational modifications of p53 during RS (Atadja *et al.*, 1995), this is a new finding and

redefines the role of p53 in SIPS, suggesting an important role for the p53/p21^{WAF1} pathway, in conjunction with pRb, in SIPS. We also proposed a general rearrangement of the gene expression networks during SIPS, involving antagonistic players in apoptosis, senescence, and cellular survival and proliferation. Therefore, we argued that the activation of DNA response pathways due to the genotoxic damage generated by H_2O_2 is the primary mechanism in SIPS.

Understanding the molecular basis of SIPS may have implications for cancer research but we do not think that SIPS and RS are representative of organismal ageing. Nonetheless, RS and SIPS may serve as biomarkers of ageing and may help understand age-related changes in stress-prone tissues and the age-related increase in cancer incidence. Some cellular studies should be useful to understand human ageing. We suggested extending research into how cells respond to external insults and signals across different mammals and in long-lived animals, and across cells derived from human donors of different ages. We also argued that the debate of RS versus SIPS is irrelevant and instead researchers should focus on understanding the mechanisms involved. Yet telomere-independent mechanisms likely prevail *in vivo* in age-related pathology.

Our results suggest changes in the stress response of WS fibroblasts when compared to normal HDFs. In addition, we suggested an uncoupling between SA β-gal and the senescent morphogenesis in WS fibroblasts. We also confirmed previous reports that WS fibroblasts are more sensitive to oxidative stress. Our research over WS led us to suggest that DNA metabolism plays a critical role in ageing. Understanding why these changes are presumably accelerated in WS may prove relevant to understand the normal human ageing process.

Based on ours as well as other recent results (Lindvall *et al.*, 2003 for arguments), we advise caution in using telomerase in anti-ageing therapies. Not only it remains unclear whether telomerase may reverse ageing but telomerase expression may alter the normal cellular functions and promote tumorogenesis. We propose developing anti-ageing interventions by identifying key players involved in the ageing process of humans, not model organisms. To that purpose, identifying biomarkers of ageing, through, for example, gene expression patterns, to accurately evaluate how aged someone is would prove priceless. Although we find lower life forms as potentially flawed models of human ageing, we do not propose that studies in these models be discarded, but rather that researchers use a sceptical open-minded approach.

We also proposed ways to integrate the modern computational approaches into ageing research. Although we find it unlikely that a full understanding of ageing may be achieved

within a near future, we argued that understanding the structure and finding key regulatory genes of the human ageing process is possible. We focused on two general ways of studying human ageing: age-related changes--e.g. at a gene expression or protein level--and changes in mammalian rates of ageing, in addition to the aforementioned progeroid syndromes such as WS. Eventually, combining information from all of these studies may allow us to build a Boolean model of the genetic network of human ageing and thus lead to the development of anti-ageing therapies. Lastly, we suggested that instead of focusing on age-related deleterious changes, researchers should also focus on the changes that occur prior to the age-related increase in vulnerability but that allow ageing to commence. Understanding these changes may be important in developing anti-ageing interventions. It is our opinion that we are at the dawn of a new era in which ageing will finally be conquered.

Appendix

In chapter 11 we presented a theoretical basis on how to apply computational approaches to study ageing. Yet we also wanted to demonstrate our strategy. Therefore, we developed a computational toolkit and used it to investigate theories of ageing in the context of comparative genomics. Although the scope of our findings are limited, we think our approach represents a first step in integrating comparative genomics in ageing research and serves as a foundation for future research.

How the genome regulates aging: development of a comparative genomics method to study human aging and a test of theories of aging (submitted for publication)

In article 1, we suggested that the mechanisms of ageing in mammals may be similar and unique. If true, then the rate of ageing in mammals is genetically controlled (Miller, 1999; de Magalhaes, 2003a for arguments). In this article, we proposed a methodology for studying the genomic basis of ageing. Our reasoning was that by employing computational tools to mine data from the genome we may be able to gather clues about the genetics of human ageing.

The first step was to develop a comparative genomics methodology and software to investigate how the genome regulates ageing. Afterwards, we tested theories of ageing in the context of comparative genomics using our methodology and computational toolkit. Our study at a comparative genomics level suggested a possible role for the Fanconi anaemia proteins in the age-related changes of murine hematopoietic stem cells. We also failed to find any evidence that mitochondria-encoded proteins play a role in regulating primate ageing--though they may be related to human longevity--and identified a putative regulatory region in the *WRN* promoter. With the ongoing avalanche of high-throughput data, our methodology may prove valuable to identify genetic players involved in the human ageing process.

How the genome regulates aging: development of a comparative genomics method to study human aging and a test of theories of aging

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Running title: Mining genomic information to study aging

Abstract

The duration of aging in humans makes it almost impossible to perform *in vivo* studies and so the genetic basis of human aging remains largely a mystery. Since the rate of aging amongst mammals is mostly determined by the genome, one promising strategy is to employ computational tools to mine information from the recent wealth of genomic data. Herein, we propose a comparative genomics method to study each type of genetic information that may regulate mammalian aging as well as an algorithm to find such information. To implement our method, we developed a computational toolkit, which we employed to test theories of aging in the context of comparative genomics. Our results fail to suggest a role for the mitochondrial DNA in determining primate rate of aging, though it may be involved in age-related pathology. We also suggest that the Fanconi anemia genes may help explain the age-related changes of hematopoietic stem cells during murine aging. Lastly, we identified a putative regulatory region in the Werner-syndrome promoter. Our toolkit is available at: http://genomics.senescence.info/software/

Keywords: aging, bioinformatics, computational biology, genetics of aging, genomics

Introduction

The crowning achievement in gerontology will be to understand human aging, the age-related process of loss of viability and increase in vulnerability that is intrinsic to all human beings [1]. Although several genes have already been shown to regulate aging in short-lived organisms [2], progeroid genes are, so far, the only identified genes that may modulate, in this case accelerate, aging in humans [3]. Finding other genes involved in human aging is of great importance. Yet due to the duration of aging in humans, there are clear technical limitations in performing *in vivo* studies and so the genetic basis of human aging remains largely a mystery.

Amongst mammals we observe a large variety of rates of aging, about 50-fold differences [4]. Humans appear to age significantly slower than most other mammals, including other primates. For instance, rhesus macaques, chimpanzees, and baboons either in the wild or in captivity age about twice as fast as humans [5, 6, 7]. The way the rate of aging varies widely from species to species, but little between populations of the same species under different environmental conditions, shows that the rate of aging is mostly regulated by the genome [8, 9]. As suggested by others, finding which genes determine rate of aging would prove crucial to develop ways to ameliorate age-related adverse effects and provide clues about what mechanisms modulate human aging [2]. Therefore, comparative genomics of aging applied to mammals can, theoretically, help researchers understand the genetic basis of aging.

Although others have argued in favor of genomics to study aging [10], much debate exists over what type of genetic information regulates aging in mammals. Besides, researchers still lack a strategy on how to employ the modern computational tools to locate such information when several mammalian genomes become available. Herein, we propose a comparative genomics method to help understand human aging. To implement our methodology, we developed a computational toolkit aimed at the comparative genomics of aging. Lastly, we tested theories of aging in the context of comparative genomics. We chose to test the two most widely accepted theories of aging: DNA damage [11] and oxidative damage derived from reactive oxygen species leakage from mitochondria [12, 13].

Results

A comparative genomics method to study human aging

Genomic differences determine rate of aging in mammals, but finding the information that controls aging is problematic since a mammalian genome holds a colossal amount of information. When compared to other primates, humans show a significantly different rate of aging despite our evolutionary proximity and genomic similarity [14]. For example, humans and chimpanzees share about 99.4% of their DNA in functionally important coding regions [15] and have essentially the same set of genes [16]. Even mice, that age 25-30 times faster than humans, share an estimated 99% of our total number of genes [17]. Consequently, the regulation of aging is located in only a fraction of the genome, as proposed by others [18]. Indeed, there is little evidence that major genomic features are involved in aging. For instance, genome size does not correlate with longevity amongst mammals [19]. Comparing the mouse and human genomes suggests that the relation between coding and non-coding regions also does not appear to play a part in aging. The mouse genome has fewer CpG islands and different patterns of G+C distribution [17], though at present there is no evidence such features are involved in aging. Although the telomeres have been implicated in aging, telomere length does not correlate with longevity in primates [20]. Therefore, we propose a three-way strategy to study the subtle genomic information that may regulate aging in mammals (Figure 1).

Phylogenetic profile of human DNA repair proteins

One hypothesis is that rate of aging differs in mammals due to different genes. For example, perhaps humans feature protective genes that short-lived mammals lack. If so, then a promising approach is to compare genomes to determine the phylogenetic distribution of genes between different species. In the context of gerontology, the aim is to determine the presence or absence of genes in different species to find trends or cohorts of genes whose presence correlates with rate of aging.

To test the hypothesis that DNA repair defects are implicated in aging [11], we generated the phylogenetic profile of human DNA repair proteins (Table 1). We based our approach on a previously published description of human DNA repair pathways [21] and on the list of 126 genes taken from the authors' website (http://www.cgal.icnet.uk/DNA_Repair_Genes.html). As detailed in the methods section, we would prefer to use only mammals. Yet given the limited

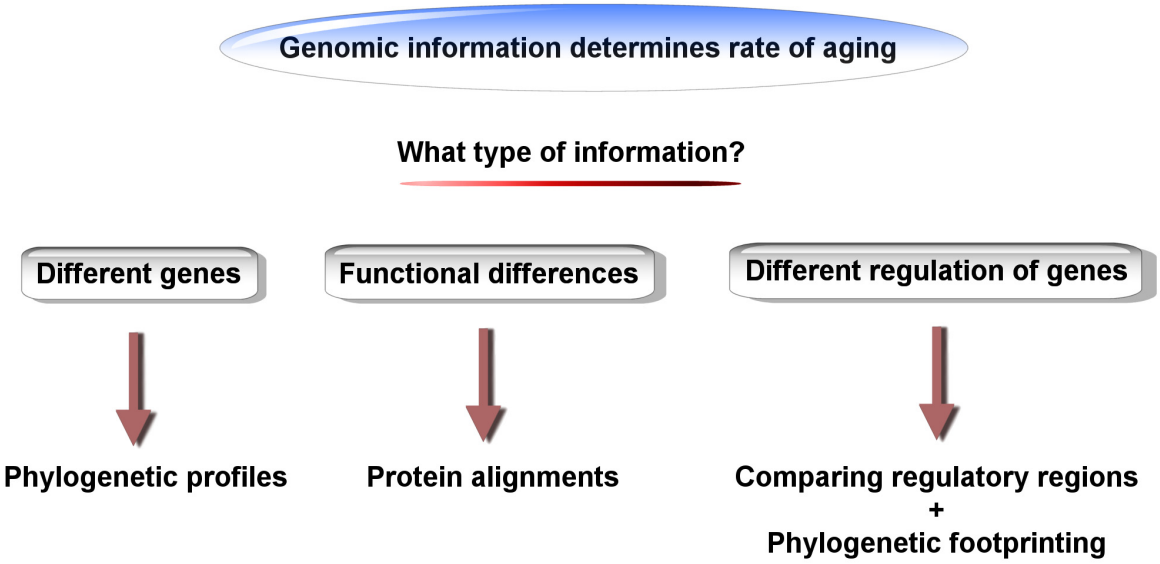

Figure 1: Strategies to identify the genomic information regulating human aging. Although the genomic information determines a species' rate of aging, we do not know for sure what type of information. It could be the simple presence or absence of genes, which would be easy to detect by way of phylogenetic profiles employing BLAST once we have enough fully sequenced mammalian genomes. Sadly, the regulation of aging will probably involve functional differences between proteins or other gene products that are harder to identify. Even so, by way of, for example, ClustalW alignments we may find conserved regions whose level of conservation correlates with rate of aging. Finally, the regulation of aging may involve differences in the transcriptional regulation of genes. Yet using, for instance, phylogenetic footprinting it may be possible to locate conserved regions in the regulatory sequences of different mammals that will allow us to find the information behind the regulation of aging.

mammalian genomes presently available, we included non-mammalian organisms as a demonstration of the method, since only hits in the mouse were carefully analyzed.

Due to possible inaccuracies in genome annotation and the limited number of mammalian genomes, our results are preliminary. Even so, it was intriguing that amidst a highly conserved protein family (>95% conservation of functional genes using our threshold, please see Table 1), mice appear to lack two proteins, FANCE (**NP_068741**) and FANCF (**NP_073562**), involved in the human Fanconi anemia. Suspected of being a progeroid syndrome [22], Fanconi anemia is a bone marrow disease and its genes appear to be involved in tolerance or repair of DNA crosslinks. FANCE and FANCF regulate the activation of the Fanconia anemia DNA repair pathway through FANCD2 [23].

Functional characterization of primate mitochondrial-encoded proteins

We know that differences between humans and other mammals result not only from different genes, but mostly from alternative splicing and differences in gene function between homologous genes. To investigate aging, it will likely be necessary to take a closer look at the gene products and so a functional characterization of proteins is necessary. Unlike functional genomics, where the aim is to identify, for instance, functional motifs, while studying aging the aim is to relate protein function or dysfunction with rate of aging. Although we also compared the DNA repair proteins of mice and humans in terms of amino acid (aa) similarity (not shown), such comparisons are unlikely to prove fruitful if only human and murine proteins are available.

Mitochondrial-encoded proteins have been implicated in aging [12] and age-related diseases [13]. To test this hypothesis, we compared the mitochondrial-encoded proteins of several organisms using the data from the NCBI website (ftp://ftp.ncbi.nih.gov/genbank/genomes/MITOCHONDRIA/Metazoa/). Our selection of model organisms was based on the criteria set in the methods section and so species evolutionary closer to humans were preferred, also taking into account metabolic and aging rates. In total, we selected the mitochondrial DNA (mtDNA) of 27 species, mostly mammals but also reptiles, birds, and amphibians, as described in the methods section. Multiple comparisons using all mitochondrial proteins revealed little information, as did those involving only mammalian proteins (not shown). We then tried to find unique or disrupted functional motifs in the proteins of, respectively, long-lived and short-lived mammals through the algorithm described in the methods section. Given that other primates age considerably faster than humans, we also investigated whether unique motifs could exist in primates or even humans that confer us

Table 1: Sample of phylogenetic profile of human DNA repair proteins representing putative functionally equivalent proteins in several model organisms. In this table, the criteria for an homolog to be considered a putative functionally equivalent protein was: be at least 60% in length and 40% in residue similar to the human reference CDS. Human and bacteria are also included in the profile as controls. We manually verified all mouse genes for which we found no hits. Calculated using BLASTP 2.2.6 and as database all non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF.

Reference protein	man	mouse	fly	worm	ecoli
TDG (410 aa):	1	1	1	0	0
MBD4 (580 aa):	1	1	0	0	0
XPA (273 aa):	1	1	1	1	0
XRCC1 (633 aa):	1	1	1	0	0
MUTYH (546 aa):	1	1	0	0	1
RAD23A (363 aa):	1	1	1	1	0
FANCF (374 aa):	1	0	0	0	0
NUDT1 (179 aa):	1	1	0	0	0
ERCC2 (760 aa):	1	1	1	1	0
XRCC2 (280 aa):	1	1	0	0	0
. . .					
OGG1 (345 aa):	1	1	1	0	0
XRCC5 (732 aa):	1	1	1	0	0
MSH3 (1128 aa):	1	0	0	0	0
RPA2 (270 aa):	1	1	1	0	0
MSH2 (934 aa):	1	1	1	1	0
ERCC3 (782 aa):	1	1	1	1	0
FANCE (536 aa):	1	0	0	0	0
MSH6 (1360 aa):	1	1	1	1	0
RAD18 (495 aa):	1	1	0	0	0
PRKDC (4128 aa):	1	1	0	1	0
Final results	125/125	120/125	85/125	67/125	5/125
Percentage	100%	96%	68%	54%	4%

delayed aging. Although we found no unique functional motifs in long-lived mammals, we found a few unique residues in human mitochondrial-encoded proteins. One example is residue 64 of NADH dehydrogenase subunit 1 or MTND1 (Figure 2A). Intriguingly, a mutation in this same residue has been hinted as a secondary mutation in Leber's optic neuropathy [24]. We also found some unique residues in primates when compared to other animals that occurred in highly conserved residues and thus liked to be functionally relevant (Figure 2B).

Transcriptional regulation and aging

Others have speculated how differences in the rate of aging amongst mammals derive from "subtle changes in the transcriptional regulation of a subset of critical genes" [25]. Indeed, evidence suggests that as much as 40% of human transcription binding sites are not functional in rodents [26]. Therefore, regulatory proteins and sequences may be the key to understand mammalian aging, as proposed by many others [18, 22].

Following the same principle described previously, we also tried to relate the mitochondrial promoter to rate of aging. Interestingly, we found 12 mismatches in short-lived mammals and none in long-lived mammals, but we found no overall correlation between regulatory motifs and rate of aging in mammals. We also focused on primate regulatory regions but failed to find any significant differences in the transcriptional regulation of mitochondrial-encoded genes amongst primates. As with proteins, there were some unique positions in the human promoter but not in putative regulatory important regions and so we assume these were false positives. Lastly, RNA genes in the mtDNA were also studied but we also failed to find any correlation between these and rate of aging in mammals (not shown).

One of the few proteins suspected of being involved in human aging is the Werner protein (WRN), which is involved in DNA repair and appears to accelerate human aging [3]. Since there is only one mammalian homologue of WRN--in the mouse--and it does not appear to be involved in murine aging, comparing multiple promoters is unlikely to be profitable at present. Therefore, we studied the human *WRN* promoter through our toolkit in search of putative transcription factor binding sites (TFBS) thought to be involved in aging (Figure 3). We found two large SP1 clusters at -386 to -225 and -783 to -668 bp from the translation start site, of which the former has been biologically determined to play a role in the regulation of *WRN* by SP1 [27].

A

```
pongo/1-318             GLLQPFADALKLFTKEPLKPSTST
macaque/1-318           GLLQPFADAMKLFTKEPLKPSTST
gibbon/1-318            GLLQPFADAMKLFTKEPLKPSTST
homo/1-318              GLLQPFADAMKLFTKEPLKPATST
gorilla/1-318           GLLQPFADAMKLFTKEPLKPSTST
chimp/1-318             GLLQPFADAMKLFTKEPLKPSTST
bonono/1-318            GLLQPFADAMKLFTKEPLKPSTST
                        *********:**********:***
ARCT output for MTND1: GLLQPFADAMKLFTKEPLKPATST
                                              ^
                        --------- ---------- ---
```

B

MTCO2

```
chimp/1-227             GDLRLLDVDNR
bonono/1-227            GDLRLLDVDNR
gorilla/1-227           GDLRLLDVDNR
homo/1-227              GDLRLLDVDNR
pongo/1-227             GDLRLLDVDNR
gibbon/1-227            GDLRLLEVDNR
macaque/1-227           GDLRLLEVDNR
dog/1-227               GELRLLEVDNR
horse/1-227             GELRLLEVDNR
bwhale/1-227            GELRLLEVDNR
fwhale/1-227            GELRLLEVDNR
hippo/1-227             GDLRLLEVDNR
sheep/1-227             GELRLLEVDNR
nzbat/1-227             GQLRLLEVDNR
jbat/1-227              GDLRLLEVDNR
mouse/1-227             GELRLLEVDNR
rat/1-227               GELRLLEVDNR
marsupial/1-228         GQLRLLEVDNR
opossum/1-235           GQLRLLEVDNR
elephant/1-228          GELRLLEVDNR
monkey/1-229            GEFRLLEVDNR
salamander/1-229        GQFRLLEVDNR
frog/1-229              GQFRLLEVDNR
turtle/1-228            GHFRLLEVDHR
falcon/1-227            GHFRLLEVDHR
buteo/1-227             GHFRLLEVDHR
alligator/1-229         GHFRLLEVDHR
                        *.:***:**:*
```

MTCYB

```
marsupial/1-381         TPPHIKPEWYFLFAYAILRSI
opossum/1-382           TPPHIKPEWYFLFAYAILRSI
chimp/1-380             TPPHIKPEWYFLFAYTILRSI
bonono/1-380            TPPHIKPEWYFLFAYTILRSV
homo/1-378              TPPHIKPEWYFLFAYTILRSV
gorilla/1-380           TPPHIKPEWYFLFAYAILRSV
pongo/1-380             TPPHIKPEWYFLFAYAILRSV
gibbon/1-380            TPPHIKPEWYFLFAYAILRSV
macaque/1-380           TPPHIKPEWYFLFAYTILRSV
monkey/1-378            TPPHIKPEWYFLFAYTILRSI
mouse/1-381             TPPHIKPEWYFLFAYAILRSI
rat/1-380               TPPHIKPEWYFLFAYAILRSI
sheep/1-379             TPPHIKPEWYFLFAYAILRSI
dog/1-379               TPPHIKPEWYFLFAYAILRSI
bwhale/1-379            TPAHIKPEWYFLFAYAILRSI
fwhale/1-379            TPAHIKPEWYFLFAYAILRSI
hippo/1-379             TPPHIKPEWYFLFAYAILRSI
horse/1-379             TPPHIKPEWYFLFAYAILRSI
nzbat/1-379             TPPHIKPEWYFLFAYAILRSI
jbat/1-379              TPPHIKPEWYFLFAYAILRSI
elephant/1-378          TPLHIKPEWYFLFAYAILRSV
salamander/1-380        TPPHIQPEWYFLFAYAILRSI
frog/1-379              TPPHIKPEWYFLFAYAILRSM
turtle/1-379            TPPHIKPEWYFLFAYAILRSI
falcon/1-380            TPPHIKPEWYFLFAYAILRSI
buteo/1-380             TPPHIKPEWYFLFAYAILRSI
alligator/1-386         TPTHIKPEWYFLFAYAILRSI
                        ** **:*********:****:
```

Figure 2: A: multiple alignment of several primates MTND1 sequence from residues 44 to 67 generated with ClustalW. Residue 64 is highlighted. The ARCT output for MTND1 represents the human sequence indicating a unique human residue ("^") as well as the conserved residues in the multiple alignment ("-").

B: multiple alignments of various animal sequences of MTCO2 and MTCYB created using ClustalW. Residues 131 to 141 of MTCO2 are show with residue 137 highlighted while residues 265 to 286 of MTCYB are shown with residue 280 highlighted. The complete set of results is available at: http://genomics.senescence.info/software/mtdna/

A computational toolkit to research the genomic basis of human aging

To automate research into the comparative genomics of aging, we developed a toolkit of Perl modules and scripts: Aging Research Computational Tools (ARCT). All of our results were obtained through ARCT, which is composed of tools for generating phylogenetic profiles and multiple alignments, determining putative TFBS, and performing phylogenetic footprinting. Included are the algorithms and statistical analysis tools described in the methods section. Simple sequence analysis tools are also included such as CpG island detection and estimation of synonymous versus nonsynonymous substitutions to search positive selection. Given our comparative biology approach, we also developed a Phylogenetic Tree Plotter (PTP) that can be used to display phylogenetic relationships of different species.

We tried to make ARCT automated, capable of dealing with large amounts of data, and easy to customize and upgrade. External links are also provided and it is easy to perform BLAST searches, or access programs like ClustalW and Gibbs through ARCT. The ARCT toolkit, documentation, and more detailed examples of ARCT are available for download on the website: http://genomics.senescence.info/software/

4. Discussion

Analysis of DNA repair genes across different taxa has been done in unicellular organisms [28]. Yet similar methods cannot be fully implemented in mammals due to the lack of sequenced genomes, and so we only compared human and mouse DNA repair pathways. Even so, the DNA repair pathways appeared well-conserved and our results do not suggest the involvement of DNA repair pathways in aging.

Our most significant finding concerned Fanconi anemia, which mostly affects the bone marrow and has been hinted as an accelerated aging syndrome [22, 23]. Intriguingly, DNA repair defects in hematopoietic stem cells have been linked with mouse aging [29] and DNA repair defects have been described in rodents when compared to humans [30]. Even though our results might derive from errors in genome annotation and sequencing, our findings may help explain the rapid age-related decline of hematopoietic stem cells in mice [31]. Mice knock-outs of other Fanconi anemia genes yield a much milder phenotype than the human disease [32]. We speculate that this occurs because mice, when compared to humans, already have a faster depletion of hematopoietic stem cell stocks due to the lack of the FANCE and FANCF proteins.

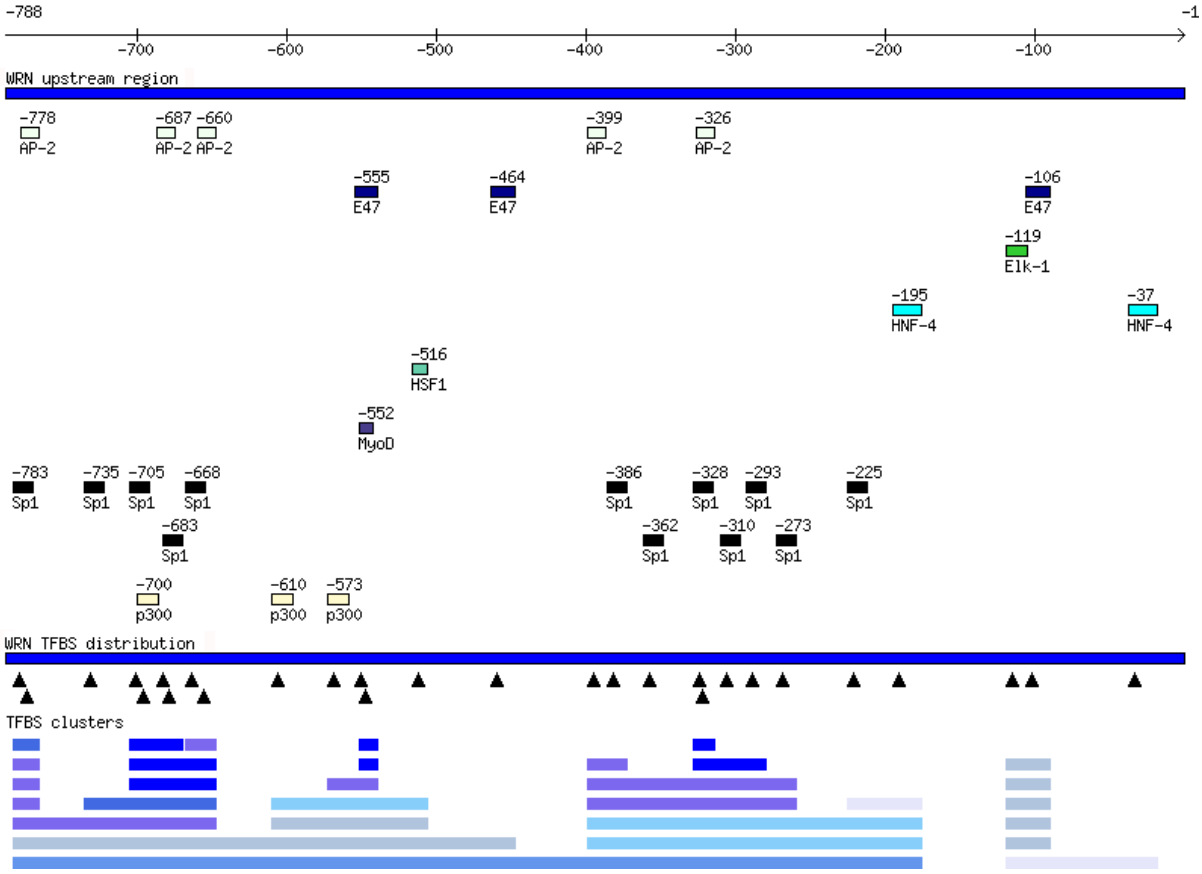

Figure 3: Putative TFBS in the upstream sequence of the human *WRN* (**NM_000553**). Note that in our algorithm the entire process is automated, from downloading the sequence from RefSeq to generating a display with putative TFBS. All the user must do is type the accession number. "-1" represents the translation start site. Each row in the TFBS cluster distribution represents the concentration of TFBS at varying thresholds.

Although, so far, expansion and contraction of protein families in mice--mostly reproduction, immunity, and olfaction--do not suggest any involvement in aging [17], phylogenetic profiles may be useful to build phylogenetic profiles of long-lived mammalian species when complete genome sequences are available, as has been done in lower life forms [28]. Even so, simple presence/absence algorithms will most likely not suffice to discover the regulation of aging in mammals.

If the mtDNA was to influence aging, we should expect to find functional differences amongst its proteins, RNA genes, or in its regulatory region. Although we cannot exclude that nuclear-encoded mitochondrial proteins and transcription factors (TF) are involved in aging, our results do not support the hypothesis that information encoded in the mtDNA plays a role in determining primate rate of aging. It was intriguing that some sequences very well-conserved throughout different metazoan classes have unique changes in primates, suggesting there may be functional differences in primate mitochondrial-encoded proteins. The mutation of Alanine to Serine in residue 64 of MTND1 has been suggested as a secondary mtDNA mutation in Leber's optic neuropathy, a hereditary, normally mid-life onset, disease [24]. It is interesting that humans are the only known primate with Alanine while most other primates feature Serine. One hypothesis is that Alanine in residue 64 of MTND1 plays a role in preventing Leber's optic neuropathy in the more long-lived primate *Homo sapiens*.

Our analyses of the *WRN* promoter revealed a previously unknown SP1 cluster of putative TFBS. Although false positives are common in TFBS algorithms, given the high density of the second SP1 cluster that we identified in the *WRN* promoter (-783 to -668 bp from the translation start site), we suggest that this cluster merits experimental verification and may also contribute to the regulation of *WRN*. Although we cannot exclude the possibility that other regions of the genome hold important information, we think comparative genomics of transcriptional regulation is one of the most promising data-mining methods to study human aging.

Through comparisons between protein families and regulatory regions amongst animals with varying rates of aging, it may be possible to determine key proteins involved in aging by, for example, locating disrupted functional motifs in proteins of short-lived rodents, or novel domain architectures in long-lived mammals, or vice-versa. In addition, if longevity evolved in primates [33], searching for positive selection of proteins appears a promising method, as has been employed [34]. Since we advocate comparisons between evolutionary close species such as mammals or even primates, we are likely to find little genetic differences and therefore the signal-to-noise ratio will be higher than employing other taxa. At present our method works

through a raw, unbiased analysis but specific analysis of motifs will be possible as soon as more mammalian genomes become available, also making it possible to research entire coding and regulatory regions.

One question is whether the aim should be to find conserved or disrupted sequences and proteins. In other words, should the differences in rate of aging amongst, for instance, primates, be related to subtle differences in key, well-conserved genes, or more widespread differences in divergent genes? Recent findings indicate that transcriptional regulator families are taxon-specific, in contrast to, for instance, the transcription initiator complex that is well-conserved amongst eukaryotes [35]. As such, we speculate that the most divergent of genes amongst primates and mammals are those involved in aging. Interestingly, the most divergent protein in the mtDNA, as estimated by aa similarity, was MTATP8 both in our selection of animals and amongst primates.

If studying human aging *in vivo* is almost impossible, then studies *in silico* may succeed where experimental approaches have failed. For instance, finding the genomic information that makes baboons, rhesus macaques, or chimpanzees age twice as fast as humans would be a major breakthrough. With the prospect of having several fully sequenced mammalian genomes within the next few years--namely, human, mouse, rat, chimpanzee, rhesus macaque, cow, pig, and dog genomes (http://www.genome.gov/page.cfm?pageID=10002154)--comparative genomics is a powerful approach to study human aging. Our method and ARCT offer a basis for future research as comparative genomics will play a crucial role in understanding the genetic basis of human aging.

Methods

Measuring rate of aging and selection of model organisms

To measure a species's rate of aging, two methods were used. Firstly, the mortality rate doubling time (MRDT) was calculated using the equation: $\mathrm{MRDT} = \frac{0.693}{\alpha}$. The α parameter derives from the Gompertz equation, $R_m = R_0 e^{\alpha t}$ where R_m is the chance of dying at age t and R_0 is the non-exponential factor in mortality [1, 5]. For example, baboons age twice as fast as humans because the MRDT for humans is between 7.6 and 8.9 years and for baboons is between 3.5 and 4.8 [7]. Although other mathematical models exist, due to frequent data limitations in studying animal aging and mammalian populations the Gompertz equation was preferred.

Secondly, physiological observations were used based on a careful review of the literature. Rhesus macaques, for instance, exhibit age-related changes in the eyes that are similar to those of humans only about twice as fast and chimpanzees aged over 35 years display several age-related changes typical of elderly humans [5, 6].

Metabolic rates were also considered, though these alone only explain the variation in rates of aging to a small extent [4]. Nonetheless, finding the metabolic basis of longevity determination may have medical applications. Uncovering what determines the progression of the genetic program--for instance, in caloric restriction-- may allow researchers to develop anti-aging interventions [36].

Evolutionary close species were preferred because their use increases the signal-to-noise ratio and the likelihood that these species share mechanisms regulating aging. Non-primate and non-mammalian species were also employed to allow the identification of functionally or regulatory relevant regions [37]. As an example, a selection of models that fit the criteria is presented in Table 2. The 27 species used in our analysis of the mtDNA were: Gorilla gorilla (**NC_001645**), Pongo pygmaeus (**NC_001646**), Pan troglodytes (**NC_001643**), Pan paniscus (**NC_001644**), Hylobates lar (**NC_002082**), Cebus albifrons (**NC_002763**), Macaca sylvanus (**NC_002764**), Homo sapiens (**NC_001807**), Balaenoptera musculus (**NC_001601**), Balaenoptera physalus (**NC_001321**), Loxodonta africana (**NC_000934**), Mus musculus (**NC_005089**), Ovis aries (**NC_001941**), Hippopotamus amphibius (**NC_000889**), Canis familiaris (**NC_002008**), Rattus norvegicus (**NC_001665**), Equus caballus (**NC_001640**), Isoodon macrourus (**NC_002746**), Didelphis virginiana (**NC_001610**), Chalinolobus tuberculatus (**NC_002626**), Artibeus jamaicensis (**NC_002009**), Chrysemys picta (**NC_002073**), Alligator mississippiensis (**NC_001922**), Falco peregrinus (**NC_000878**), Buteo buteo (**NC_003128**), Andrias davidianus (**NC_004926**), Xenopus laevis (**NC_001573**).

Building phylogenetic profiles

The first step in building phylogenetic profiles was to identify the presence or absence of homologs across different species. Homologs of each human CDS were identified by searching multiple databases using BLAST. For comparisons between closely related species, BLAST is a reliable algorithm [38] and the loss of sensitivity is compensated by its speed [39].

Mouse homologs were then analyzed. Since only less than 1% human genes fail to have an homolog in the mouse [17], a stringent threshold was used to identify hits, defined as human proteins lacking a functional equivalent in the mouse genome. One criterion was the 1:1

Table 2: Suggested model organisms for studying aging at a genomic level. Last common ancestral represents estimates--often still under debate--of when a species and humans split, in millions of years ago (Mya). References: [5, 6, 7, 33, 48].

Species (last common ancestral)	**MRDT in years**	**Observations**
Humans	7.6-8.9	
Chimpanzees (5.4 Mya)	Similar to humans	Our closest relatives, which age surprisingly faster than humans
Other primates (23 Mya)	3.5-4.8 (baboons), 8 (rhesus macaques)	As with chimpanzees, some other primates appear to age about twice as fast as humans
Mice and rats (91 Mya)	0.3	Two of the fastest aging mammals
Bats (92 Mya)	3-8	Featuring a slow rate of aging for their size and metabolism
Some common mammals from farm to domestic animals (92 Mya)	3 (dog), 4 (horse), 1.5 (sheep)	
Long-lived mammals such as elephants and whales (92 Mya)	8 (elephants)	
Slowly aging reptiles (200 Mya)	Often no MRDT detected	Since mammals evolved from slowly aging reptiles, it may be useful to research reptilian species
Long-lived birds such as gulls, fulmars, or parrots (310 Mya)	>6	Although evolutionary further from us than reptiles, birds share our high metabolic rates and might be useful in the study of human aging

ortholog, since only about 80% of human and murine genes are 1:1 orthologs [17]. In addition, other criteria were: global amino acid identity (*i*), aa conservation (*c*), $\frac{\text{length of alignment between both sequences}}{\text{length of query sequence}}$ (*l*), or a combination of these criteria--e.g. *il*. Different criteria were used to obtain different distributions of putative functional related proteins for different thresholds (in the range 0.4 to 0.8 for each criterion), which allowed us to focus on the protein groups most divergent/convergent. Although ARCT was used to automate all these processes, each hit was manually confirmed by a comprehensive review of the literature.

Algorithm for locating aging-related genomic regions

For *n* sequences of the same protein from multiple organisms (Seq_1, Seq_2 . . . Seq_n), a multiple alignment was generated: Aln_{all}(Seq_1 to Seq_n). Aln_{all} may include, for instance, animal species from different classes, only mammals, or only primates. For a given sub-group of organisms, a novel alignment was generated as well as an alignment of the reference sequences minus those in the sub-group, respectively: Aln_{qr}(Seq_x, Seq_y, Seq_z . . .) and $Aln_{all\text{-}qr}$. There may be only one query sequence (e.g. a human sequence Seq_h) in which case Seq_h was used instead of Aln_{qr}.

To find disrupted sequences, the algorithm first found mismatches between the query sequences Aln_{qr}. The rationale being that if degeneration occurred, conservation would be absent in comparisons between sequences taken from animals of different families. Then non-conserved positions were matched against the non-conserved positions in $Aln_{all\text{-}qr}$. A position conserved in $Aln_{all\text{-}qr}$ that is a mismatch in Aln_{gr} was assumed as a degenerate position in a functionally important region.

A complementary strategy was to find novel functional motifs. Conserved positions in Aln_{qr} were compared with $Aln_{all\text{-}qr}$. If these were not conserved in $Aln_{all\text{-}qr}$ or conserved but with a different residue or nucleotide, a novel functional motif was assumed to had evolved. If Aln_{qr} are closely related or if there is only one query sequence, then only positions conserved in $Aln_{all\text{-}qr}$ but with a different residue in Aln_{qr} were assumed to be novel and functionally relevant; the rationale being that closely-related query sequences cannot be assumed to have novel functions unless these have been demonstrated to be functionally important.

The algorithms used were the ClustalW 1.83 [40] multiple alignment algorithm and the motif sampling algorithm Gibbs [41] implemented through ARCT.

In general, the threshold for finding a disrupted residue was that it must be a mismatch in Aln_{qr} but not in $Aln_{all\text{-}qr}$ and a single residue must be conserved in at least 30% of the sequences in $Aln_{all\text{-}qr}$. Yet if the similarity of the query sequences is above 90%, then weak matches are also considered as mismatches in Aln_{qr}. For finding novel functional residues, a sole residue must be in at least 60% of the query sequences, though adjustments for highly divergent or well-conserved sequences are calculated. Since these are mostly arbitrary values, they may also be modified by the user. The novel residue found in Aln_{qr} may not occur in more than 10% of the sequences in $Aln_{all\text{-}qr}$. Finally, the strategy for investigating nucleotides is similar but a more stringent threshold is used since false positives are more likely to occur. Normally, a 20-30% adjustment is used, but that can be modified by the user.

Studying transcriptional regulation

Position weight matrices were taken from TRANSFAC version 6.0 [42] to build a database of 23 transcription factors (TF) thought to be involved in aging: AP-1, AP-2, C/EBP, CRE-BP1/c-Jun, CREB, E2F, E47, Elk-1, HNF-3, HSF1, Max, MyoD, NF-AT, NF-κB, SRF, STAT1, STAT3, Sp1, TATA, c-Myb, c-Myc/Maxi, p300, p53. All these matrices are available on our website as well as a literature review of the importance of each. Included in ARCT are tools to search for the corresponding TFBS--the list is, of course, fully customizable.

The algorithm for finding putative TFBS involves a scoring system that gives a histogram of the TFBS found on each segment. For a given sequence of length l, there are $\frac{l}{100}$ thresholds, so the histogram only works for large sequences ($l > 200$). For each threshold, clusters are established according to the $\frac{n_{TFBS}}{l}$ ratio being n_{TFBS} the number of putative TFBS found. The threshold determines the size of the allowed clusters, so the histogram contains clusters of different sizes. The highest scoring cluster is then used as reference to normalize the others, which are drawn in different shades of blue.

Aging Research Computational Tools (ARCT)

All programs were developed in Perl making use of the Bioperl [43] and TFBS [44] modules. Online programs were developed using the CGI module. The default criteria for a CpG

island is a 500-bp stretch of DNA with a C+G content of 55% and an $\frac{\text{observed CpG}}{\text{expected CpG}}$ in excess of 0.65, as described in [45].

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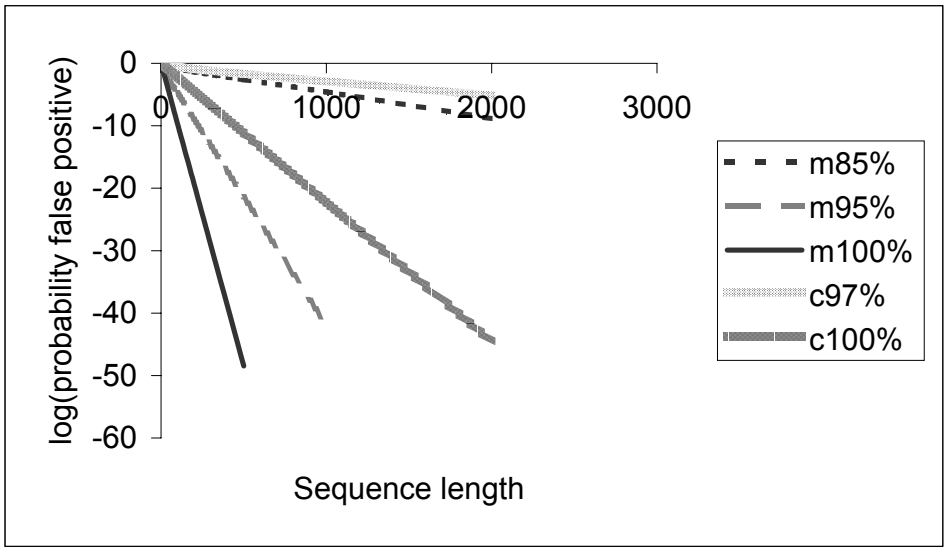
Addendum: Estimation of false positives and possible solutions

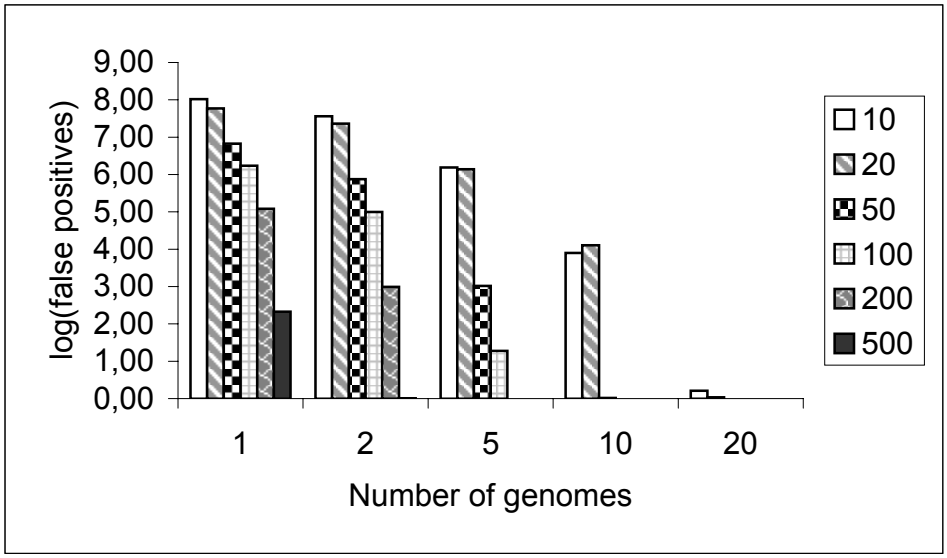
The comparative genomics of aging has the major problem of trying to find subtle differences when the human genome has about three billion base pairs. Based on the size of the human, mouse, and chimpanzee genomes and their predicted nucleotide similarity [14, 17], we calculated the chances of finding false positives through comparative genomics depending on the length of the sequence and the similarity used as threshold (Figure 4A). Considering the three billion base pairs in the human genome, our results suggest that genome-wide comparisons between the human and chimpanzee genomes is bound to fail since even looking for sequences with 100% similarity we are likely to find false positives of over 300 base pairs. Even using a combination of the mouse and the chimpanzee genomes at a stringent threshold we are likely to find false positives if we search sequences below 100 base pairs. Besides, not only genetic information regulating aging is probably a small fraction of the genome but it is hidden amongst a variety of noises, and we are not strictly looking for conserved regions to identify function but rather to correlate the genomic information with rate of aging. It would require several mammalian genomes (>20) to find the information in question through genome-wide scans (Figure 4B). Therefore, we must refine our searches.

We can restrict our analysis to coding regions, which involve an estimated 60 million bp or 20 million amino acids [46]. Amino acid similarity between human and mouse proteins varies considerably and it may be possible to find trends correlating these changes with aging [17]. A similar approach is to search regulatory regions. These can be defined, for instance, as 2,000 bp upstream of genes since, although regulatory regions tend to be closer to the transcription start site, due to possible annotation errors it is recommended the use of a large range. Assuming 30,000 human genes gives 60 million nucleotides, which, like searching through coding regions, greatly increases our data-mining odds. Obviously, our chances of finding false positives decrease if we are trying to find a needle in a smaller haystack. Interestingly, if we are looking for subtle genomic differences then primate genomes allow us to use a more stringent threshold and thus decrease the likelihood of false negatives but reference sequences from other mammals are also necessary to identify relevant regions.

Lastly, by taking into account our knowledge about aging, we can focus on pathways previously implicated in aging and derive specific hypothesis. Unbiased genome-wide screens may be possible when several mammalian genomes are available. At present, however, comparative genomics is more useful if used in a Bayesian context.



A



B

Figure 4: A: Probability of finding a false negative in the mouse (small lines) or chimpanzee genome (large lines) while searching for conserved sequences. Thresholds represent nucleotide similarity between the human and mouse (85, 95, and 100%) or human and chimpanzee (97 and 100%) genome. Nucleotide similarity was assumed to be 80% between the mouse and 95% between the chimpanzee and the human genome [14, 17]. These values represent overall genome comparisons and since specific genomic regions--e.g. coding regions--are not taken into consideration they should be seen as approximations to the real biological values.

B: Number of false positives according to sequenced genomes based on the probability of finding a 95% sequence match for genomes with three billion nucleotides with an average 90% similarity with the human genome. Obviously, these are estimates of sequence similarity and so our results are approximations of the real biological values.

We employed the cumulative function of the binomial distribution, so that the chances represent the probability that there is a false positive of at least a given similarity. The probability (p_{seq}) that a sequence of length (l) will be a certain threshold (thr) similar to its homologous sequence in another organism can be calculated using the binomial distribution [47]: $p_{seq} = \left(\frac{l!}{(l-m)!m!} \right) d^m (1-d)^{l-m}$ in which m is the number of mutations, $l(1 - thr)$, and d is the degeneracy or 1 minus the overall similarity between the two genomes. False positives can be estimated using the cumulative binomial distribution: $P_{seq} = \sum_{m=0}^{m} p_{seq}$

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Rôle des télomères dans la sénescence induite prématurément par les stress et design de stratégies anti-vieillissement

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Résumé

La durée de vie humaine normale fait que les études gérontologiques doivent être basées sur des modèles comme des animaux et des cellules. La sénescence réplicative et la sénescence induite par les stress (SIPS) sont deux modèles cellulaires partageant de nombreuses caractéristiques communes. Bien que les télomères jouent un rôle majeur dans la sénescence réplicative, leur implication dans la SIPS n'est pas claire.

Dans ce travail, nous voulons tout d'abord réfléchir sur la validité des modèles de vieillissement humain. Nous avons publié un nouveau modèle de l'évolution du vieillissement humain. Ce modèle offre une vue plus précise de l'évolution du vieillissement humain et suggère que les études sur modèles humains devraient être favorisées, bien que des études sur d'autres mammifères, des reptiles et des oiseaux peuvent s'avérer utiles.

Deuxièmement, nous voulons élucider l'importance des télomères dans la SIPS et étudier l'expression génique des cellules en SIPS. Utilisant des fibroblastes humains immortalisés par la télomérase, nous n'avons trouvé aucune évidence que les dommages spécifiques aux télomères sont à l'origine de la SIPS. Dans notre modèle publié, ni le TGF-β1, ni un raccourcissement critique des télomères ne semblent jouer un rôle crucial dans la SIPS. Nous suggérons que des dommages généralisés à l'ADN sont à l'origine de la SIPS et proposons un réarrangement dans les réseaux d'expression génique dû au stress. Nos travaux suggèrent aussi la plus grande prudence pour toute thérapie anti-vieillissement basée sur la télomérase.

Enfin, nous avons publié des stratégies pour l'intégration des approches bioinformatiques en biogérontologie. Bien qu'une résolution du problème du vieillissement nous paraisse très éloignée, nous défendons qu'il sera possible d'identifier des génes-clés dans le vieillissement humain.